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FURTHER EXPERIENCES WITH STREPTOMYCIN THERAPY IN UNITED STATES ARMY HOSPITALS

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MEDICAL CORPS, UNITED STATES ARMY

EVALUATION of streptomycin was begun in United States Army Hospitals in 1945. This investigation is still in progress. A special research unit, established originally at Halloran General Hospital, is now continuing to function at Brooke General Hospital in San Antonio. Here sensitivities of bacteria to streptomycin have been tested and the findings correlated with studies on absorption, distribution, and excretion of the drug in the body following parenteral administration.[3] This unit, in addition, is carrying on intensive studies on certain types of infections, especially surgical. Data on these and on all patients treated with streptomycin in U. S. Army Hospitals are recorded on special forms for permanent retention. It is our purpose here to present in summary form these cumulative experiences, based on an analysis of 1,200 cases.

A complete understanding of the properties of streptomycin is an absolute necessity if the drug is to be used intelligently. The principal characteristics[15] are as follows: (1) Streptomycin is an organic base freely soluble in water but not in the common organic solvents. (2) Solutions of this compound are remarkably stable, both chemically and biologically. Refrigeration is optional for short periods of storage. (3) In contrast to penicillin, streptomycin is not destroyed by enzymes or by bacteria. (4) The drug is standardized so that 1 "S" unit is approximately equivalent to 1 microgram. (5) A wide variety of pathogenic gram-negative and gram-positive bacteria are susceptible in vitro to streptomycin (Table I). Streptomycin is highly selective in its antibacterial activity, different strains showing marked variation in their sensitivity to the drug, and naturally drug-fast strains are encountered in nearly all species. (6) The activity of the drug is depressed by low pH, dextrose, high concentrations of inorganic salts, and reducing substances. (7) Clinical response to this antibiotic is closely related to the in vitro sensitivity of the bacteria. Infections caused by organisms with high in vitro sensitivities may respond favorably; whereas those with low sensitivities are usually refractory to treatment. (8) Absorption, excretion, and distribution of streptomycin generally follow the same pattern after parenteral administration as penicillin. The exceptions are that no absorption into the general circulation takes place following administration orally or by nebulization. (9) Bacteria acquire fastness to

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streptomycin more rapidly than to penicillin. (10) This drug is relatively nontoxic when given for short periods of time. Toxicity is commonly observed when the courses of therapy are prolonged.

TABLE I. BACTERIA SUSCEPTIBLE IN VITRO TO STREPTOMYCIN

Aerobacter aerogenes	Pasteurela tularensis
Alkaligenes faecalis	Proteus morganii
Brucella abortus	Proteus vulgaris
Brucella melitensis	Pseudomonas aeruginosa
Eberthella typhosa	Salmonella species
Escherichia coli	Shigella species
Hemophilus ducreyi	Streptobacillus moniliformis
Hemophilus influenzae	Actinomyces bovis*
Hemophilus pertussis	Bacillus anthracis*
Klebsiella ozaenae	Corynebacterium diphtheriae*
Klebsiella pneumoniae	Diplococcus pneumoniae*
Neisseria gonorrhoeae*	Mycobacterium tuberculosis
Neisseria intracellularis*	Staphylococci*
Pasteurella pestis	Streptococci, hemolytic*
	Streptococci, nonhemolytic

Streptomycin is ineffective clinically against anaerobic, mycotic, protozoan, viral, and spirochetal infections, and worm infestations. Rickettsiae of epidemic and murine typhus, Rocky Mountain spotted fever, and rickettsialpox are sensitive in vitro to streptomycin.

*Penicillin-sensitive.

RESULTS OF THERAPY

The following are illustrative of types of infections which, in our experience, have shown various degrees of response to streptomycin.

Infections of the Urogenital Tract.—Four hundred and sixty-five patients with infection of the urinary tract were submitted to critical analysis and study.[12] All patients had a complete bacteriologic survey of the urine prior to treatment. Culture-sensitivity tests revealed that streptomycin had a bacteriostatic effect in vitro on 87 per cent of the bacteria recovered before treatment, while the remaining 13 per cent were naturally drug-fast. Seventy-nine per cent of the microorganisms isolated were gram-negative bacilli. The remaining 21 per cent were gram-positive cocci, a third of these nonhemolytic streptococci. The survey showed that if bacteria are not inhibited in vitro by a concentration of 16 μg per cubic centimeter, the chances of eliminating them by streptomycin therapy are not good. Streptomycin intramuscularly administered can maintain a concentration in excess of 16 μg per milliliter of blood serum. The drug is excreted in very high concentrations in the urine (1,000 μg per milliliter or more). This suggests that streptomycin acts primarily through delivery by way of the blood stream to the tissues and not through the urine.

Of the four hundred and sixty-five patients treated, an outright cure was obtained in 34 per cent of the patients; an additional 21 per cent were improved, that is symptoms were ameliorated, but without complete bacteriologic remission. The remaining 45 per cent were not benefited. As experience was gained concerning the factors which limit successful streptomycin therapy in urinary tract infections, proper selection of cases led to a progressively higher rate of cures. Best results were obtained when infections were caused by *Escherichia coli*. Failures were especially frequent when the causal organisms were *Pseudo-*

monas aeruginosa and *Streptococcus faecalis*. There was no appreciable difference in results of therapy between monobacterial and polybacterial infections, provided that all organisms were susceptible in vitro to streptomycin. The marked constitutional signs and symptoms accompanying acute pyelonephritis or acute exacerbations of chronic pyelonephritis responded dramatically to streptomycin; mild urinary tract symptoms and low-grade fever responded more irregularly. Patients with paraplegia were benefited by streptomycin therapy, although the effects were rarely permanent except in patients with closed urinary systems and automatic bladders. In these patients clinical remissions were not infrequent in spite of the fact that complete sterility of the urine was not achieved. Presence of obstructive calculi precluded a successful chemotherapeutic result. Advanced renal pathology, undrained abscesses, presence of neoplasms, and infections in the prostate were other causes of failure of streptomycin therapy. Drug-fastness is a constant feature in cases in which the prescribed course of therapy has not sterilized the urine. No advantage accrued from the administration of a second course of the drug if the first course had failed to sterilize the urine. Local instillations of streptomycin into the urinary tract via urethral catheters, cystostomy, or pyelostomy tubes have been found to be of no value in our experience.[7]

A dosage of 1.2 to 2.4 Gm. per day of streptomycin in three or four hourly divided doses for three to five days is recommended for treatment of the average infection of the urinary tract due to susceptible organisms. Alkalinization of the urine with streptomycin therapy may be advantageous in selected cases.[5] There is no contraindication to the concurrent use of penicillin or sulfonamides with streptomycin if warranted by the clinical and laboratory findings. The percentage of satisfactory results is highest when the requirements of (1) free flow of urine, (2) susceptible organisms, and (3) adequate dosage of the drug are met.

Streptomycin may be a useful agent in uncomplicated nongonococcic urethritis of bacterial origin and in acute epididymitis when the organisms are susceptible.[9] Uncomplicated gonorrheal urethritis responds to streptomycin as dramatically as to penicillin, except when there is prostatic involvement. In tuboovarian disease, particularly disease of less than one year's duration, the antibiotic seems of value in those instances in which penicillin and the sulfonamides no longer are effective. The drug has a place in the management of sinus tracts of urinary tract origin infected with streptomycin-sensitive organisms but will not eliminate surgery in most instances. In patients with Reiter's syndrome and amicrobic cystitis of unknown etiology, no consistently beneficial effects have been observed with streptomycin therapy. Neither parenteral nor oral administration can produce an effective concentration of streptomycin in prostatic secretions, which explains why this compound has not proved beneficial in the treatment of prostatis.[9]

Tularemia.—Ten patients with tularemia were treated with streptomycin, one with the oculoglandular type, one with the typhoidal type, and the others

with the ulceroglandular type.[8] Pneumonia was a complication in three patients, in one of whom it was associated with nephritis. Streptomycin was administered to all patients intramuscularly at intervals of three or four hours in an average daily dose of 2 Gm. for periods of seven to fourteen days. Recovery was smooth in all patients, without relapses or complications. The most dramatic responses were obtained in patients with the typhoidal type of infection and with early ulceroglandular tularemia complicated by pneumonia. Infection in suppurating lymph nodes treated by aspiration and followed by one or two instillations of streptomycin subsided rapidly. Healing of open lesions in ulceroglandular tularemia was slow but progressive. The rate seemed related to the duration of the infection. The proposed local injection and topical applications of streptomycin to the ulcerated buboes to provide therapeutic concentrations of drug in relatively avascular tissues remain to be fully evaluated. These experiences support the conclusion of others that streptomycin is the most effective agent now available for the treatment of tularemia.[1, 5]

Bacteriemia.—Since 1943 the superiority of penicillin over all other available forms of treatment for bacteriemias due to susceptible gram-positive organisms has been clearly demonstrated. Gram-negative bacilli, which are usually resistant to penicillin, also may on occasion invade the blood stream with serious consequences. The mortality rate from gram-negative bacteriemias is not as high as in the case of gram-positive coccal bacteriemias and the fatalities have been reduced further with the use of the sulfonamides. Streptomycin therapy promises to be even more efficient. The advantages lie in the prompt clinical responses and in the relatively trivial untoward reactions encountered with short courses of therapy.

Thirty-three patients with bacteriemia were treated with streptomycin in U. S. Army Hospitals.[10] In the majority of these the infection arose in the urinary tract; other foci of infection in decreasing order of occurrence were sepsis in the peritoneal cavity, the female genital organs, and bone, including the middle ear and mastoid. Penicillin and/or sulfadiazine failed in twenty of the thirty-three patients before streptomycin therapy was employed. The dosage of streptomycin was between 2 and 4 Gm. a day in divided intramuscular doses, and treatment was continued for an average period of fourteen days. Thirty patients recovered and three died. The deaths in each instance were caused by advanced sepsis. Beneficial results were attributed to streptomycin in twenty-six of the thirty patients, and in the four remaining patients the results were of questionable value. In one of these the blood stream was cleared, but sepsis was unrelieved until surgical drainage of an abscess in the kidney was effected. In the second patient with bacteriemia also originating in the kidney, blood cultures for *Klebsiella pneumoniae* were again positive after seven days of treatment. There was no response to a second course of the drug, and in all probability the bacteria had become resistant after the first course of therapy. There were no unusual features in the other two cases. One observation is common to the four failures: surgical treatment of the primary focus was not accomplished in conjunction with the chemotherapy.

In nearly every instance temperature subsided by a stepladder type of lysis. In most instances the blood was rapidly sterilized of the bacteria, with surgical drainage contributing to the most rapid clearing of the blood stream. Clinical improvement was usually evident by the third or fourth day of treatment. With urinary sepsis the bacteriemias cleared rapidly, but pyuria continued until the foci of infection were removed. In cases of bone lesions the infection was localized by streptomycin, but surgical intervention was necessary in the management of the primary focus.

Laboratory studies show that an additive effect is obtained on bacteria from the use of subinhibitory concentrations of streptomycin and penicillin.[6] Competitive excretions result in slightly higher drug levels when both agents are parenterally administered simultaneously. Streptomycin therapy alone or in combination with penicillin is warranted in the occasional instance of bacteriemia due to gram-positive cocci where a favorable response to maximal doses of penicillin is not obtained. Streptomycin is indicated also in the therapy of subacute bacterial endocarditis due to penicillin-fast nonhemolytic streptococci and gram-negative bacilli. Therapy must be continued beyond three weeks, and one must be prepared to accept the risk of labyrinthine disturbances from streptomycin toxicity developing in the occasional patient. On the basis of these experiences, the prerequisites for successful streptomycin therapy of bacteriemia are that: (1) the organism is sensitive to the drug; (2) acute endocarditis has not yet developed; (3) accessible foci of infection are adequately drained; and (4) the dosage of streptomycin is at least 2 Gm. per day; the interval between doses is spread so as to provide bacteriostatic blood levels; and the duration of treatment is long enough.

Infections Involving the Central Nervous System.—Streptomycin is now regarded as the drug of choice in the treatment of bacterial meningitis due to susceptible organisms.[1] The results in the U. S. Army series,[13] in general, have been very favorable, especially when treatment was started early in the disease. Of sixteen patients treated, twelve responded favorably and recovered, while four died. Deaths were due to: (1) use of streptomycin for in vitro resistant *Cryptococcus hominis* infection; (2) advanced sepsis; (3) loculation of pus in the base of the skull in two cases. It is important to remember in the management of the patient with meningitis that streptomycin, like penicillin, diffuses poorly into the cerebrospinal circulation following parenteral administration, and that intrathecal injections must be combined with the intramuscular route of administration. The sulfonamides are the only effective bacteriostatic drugs which pass the blood meningeal barrier in effective concentrations. For this reason it may be desirable to combine oral sulfadiazine with streptomycin and/or penicillin therapy.

The scheme of treatment employed in the treatment of meningitis in adults is as follows: When the diagnosis of meningitis due to gram-negative bacilli has been established, 50 to 100 mg. (average dose, 1 mg. per kilogram) of streptomycin dissolved in 10 c.c. of sterile physiologic saline are administered immediately by the intrathecal route. The injection is repeated every twenty-four hours until the patient has recovered and negative cultures have been

obtained. In addition, 0.5 Gm. of streptomycin is administered intramuscularly every four hours. Treatment with sulfadiazine and penicillin may be carried on simultaneously. The total duration of treatment with streptomycin should not exceed seven days.

Streptomycin occupies a place similar to penicillin in the chemotherapy of extradural and solitary brain abscess when caused by susceptible gram-negative or polybacterial infections.[13] The technique of aspiration of pus through a trephine followed by injection of the drug (1 per cent saline solution) through a catheter produces satisfactory results except where bone is involved and sequestrectomy is required. Systemically administered drug should be given simultaneously with the local instillations to protect against dissemination and spread of the infection. It is emphasized that streptomycin does not reach the brain in assayable amounts and that therapeutic levels at the site of the infection can be achieved only by direct injection. Of eight patients with solitary brain abscess with varied bacteriology and primary causes receiving surgical drainage and/or wound revision, together with combined systemic and local streptomycin therapy, recovery occurred in seven. The eighth patient died of sepsis.

Suppurative Infections of the Ear and Mastoid.[13]—Five cases of otitis externa due to susceptible gram-negative bacilli and gram-positive cocci responded favorably with three or four applications daily of cotton packs soaked in 1 per cent sterile aqueous streptomycin solution. Chronic otitis media of mixed bacterial etiology also was treated with topically applied streptomycin, together with parenterally administered drug in a few instances. Of nineteen patients seventeen were benefited, while in two instances of *Ps. aeruginosa* infection no improvement resulted. Combined therapy seems rational because of the possibility of not contacting all reaches of the infection by either route alone Five to seven days of treatment seem adequate for the majority of cases. Neither local nor systemic toxic phenomena have been observed from topical applications of streptomycin to the ear.

There may be a place for streptomycin alongside penicillin in the surgical and postoperative management of mixed infections of the mastoid. A patient with mastoiditis with lateral sinus thrombosis recovered without sequelae after eventration of the clot with parenteral streptomycin protection.

It is emphasized that streptomycin therapy can be a valuable addition to the therapeutic armamentarium for infections of the ear, meninges, and brain only when the organisms are susceptible to the drug and when sound proved surgical principles are strictly adhered to. There is no contraindication to the concurrent use of penicillin or sulfonamides and streptomycin.

Infections of the Respiratory Tract.—Pleuropulmonary infections in which gram-negative bacilli are implicated have been treated with sulfonamides and type-specific antisera with inconstant success. These forms of therapy, therefore, leave much to be desired. Streptomycin has been evaluated in predominantly gram-negative and other types of lesions involving the respiratory tract.[1, 2, 5]

Six patients with lobar pneumonia in which *K. pneumoniae* were the predominating organism in the sputa were treated with streptomycin. In each instance initial treatment was empirical, with penicillin alone or in combination with sulfadiazine. Each patient remained critically ill in the face of this therapy. After sputum cultures showed *K. pneumoniae* predominating, a change was made to streptomycin therapy. The dosage employed ranged from 0.2 to 0.4 Gm. given intramuscularly every four hours for an average of ten days. Response was striking in all instances, the temperature and pulse having returned to normal limits in seventy-two hours in three instances. There were no relapses. Complete clearing of the lungs, however, varied according to the duration of the infection. Certainly on the basis of this limited experience, streptomycin therapy is indicated in pneumonias caused by the Klebsiella organisms. Two cases of persistent *Hemophilus influenzae* infections in penicillin treated pneumonias showed rapid resolution on streptomycin therapy. A patient with a hemolytic streptococcus pneumonia was developing progressive clinical signs in spite of seventeen days of normally adequate penicillin. The penicillin was discontinued and streptomycin, 0.25 Gm. intramuscularly every three hours, was given with beneficial results. Partial resolution was noted clinically and by x-ray in eight days, and complete resolution two days later. Another patient, who developed postoperatively a type IX pneumococcus pneumonia with bacteriemia, showed no response whatever to streptomycin. The organism was drug-fast. Therapy with a combination of penicillin and sulfadiazine was successful. In two children, no dramatic alteration of the course of the disease attended streptomycin therapy in pertussis with bronchopneumonia. Streptomycin was of no value in the treatment of one patient with atypical pneumonia.

Gram-negative bacilli are infrequent primary causes of empyema. They usually are present in mixed culture with the more commonly causal gram-positive cocci. The treatment of pure coccal empyema by intrapleural injection of penicillin and by surgery has yielded very satisfactory results. Streptomycin has been tried in only a small series of patients, either alone or in combination with penicillin therapy. The results as a whole are not spectacular. This, in all probability, is due to the fact that streptomycin activity is markedly inhibited in exudates which are acid in reaction. It seems important to emphasize the fact that in all the cases in which improvement occurred the drug was employed in conjunction with adequate surgical drainage. A possible advantage of the combination of streptomycin with penicillin in empyema may be the more effective action of penicillin on gram-positive organisms, by virtue of the suppressive action of streptomycin on penicillinase producing gram-negative bacilli. In two instances of lung abscess and one instance of bronchiectasis, a combination of penicillin and streptomycin, administered by nebulization and the intramuscular route, was considered beneficial. Three patients with bronchiectasis were treated only by nebulization and parenteral streptomycin without effect. Two patients with pleuritis, one of undetermined origin and the other secondary to peritoneal suppuration, also were treated. There was no beneficial effect in the former patient. Improvement resulted in the latter after the peritonitis was controlled by combined streptomycin and penicillin therapy.

Two patients treated prophylactically after receiving stab wounds of the abdomen and chest did not become infected.

One case each of blastomycosis, actinomycosis caused by *Nocardia asteroides*, and moniliasis was treated with streptomycin. Sustained improvement and remission lasting for an eight months' period of observation were observed in the patient with blastomycosis. The patient with actinomycosis showed remission only while under treatment. The organisms became drug-fast and the disease progressed when therapy was discontinued. No beneficial effect from streptomycin therapy was noted in the patient with moniliasis. Six patients with chronic bronchitis receiving streptomycin and/or penicillin alone or in combination both by the aerosol and intramuscular routes showed elimination of the gram-negative organisms and temporary clinical improvement which was sustained only in those receiving both penicillin and streptomycin. Asthma, Hodgkin's disease, pulmonary sarcoidosis, and other conditions of unknown etiology were not benefited by streptomycin. (Asthma, two patients; pulmonary sarcoidosis, seven; Hodgkin's disease, two; acute pericarditis and rheumatic heart disease, one each).

Brucellosis.[11]—Twenty-nine patients with brucellosis have been treated with streptomycin, of whom sixteen had acute and thirteen had chronic cases. Blood cultures were positive for Brucella in fourteen of the sixteen patients with acute cases and a bacteriemia was present in two with chronic cases. The organisms were very sensitive in vitro to streptomycin. The dosage of streptomycin varied between 1 to 2 and 6 Gm. daily (two patients) for an average period of fourteen days. None of the patients with "chronic" brucellosis received any benefits whatsoever from streptomycin therapy. Of those with acute cases, only two of the twelve treated with streptomycin alone had fairly prompt remissions and negative subsequent blood cultures. The bacteriemia disappeared in some but not in all of the subjects while the drug was being administered. Exacerbations occurred in five of the twelve patients.

Detailed study of one of the patients under treatment led to the support of the hypothesis that the foci of infection in brucellosis were not penetrated by streptomycin administered parenterally. Oral sulfadiazine, which earlier in the patient's disease was found to be ineffective, was given in addition to the parenteral streptomycin. A prompt response was obtained and the patient has remained well for eighteen months. Five additional patients with acute brucellosis (positive blood cultures for Brucella in four) also received combined streptomycin (3 Gm. daily) and sulfadiazine (6 Gm. daily) for an average period of fourteen days. Two patients are symptom-free at this writing. There were recurrences in two others six and twelve weeks, respectively, after therapy was discontinued. The fifth patient, a 24-year-old woman from whom no Brucella were cultured, was unaffected by the combined therapy.

Infections of Intestinal Origin.—

Typhoid Fever:[11] Six patients with typhoid fever were treated. The combined oral and parenteral route was used in three patients, and the parenteral route of administration alone in the other three. No remarkable results were achieved. In only one patient, a 5-year-old child who was given the usual adult

dosage (proportionately three times as great a dose as given the other patients), did the fever end abruptly enough to suggest a response to streptomycin therapy. In two patients who were asymptomatic typhoid carriers, combined oral and parenteral streptomycin therapy did not eliminate typhoid bacilli from the feces. A third carrier, a recent patient with typhoid fever with typhoid periostitis and positive bile cultures, was benefited by resolution of the bone lesion and elimination of *Eberthella typhi* from all cultures.

Shigella and Salmonella Infections:[11] Beneficial effects were noted in ten patients with bacillary dysentery caused by *Shigella sonnei* in four and by *Shigella flexneri* subtypes in six, the majority having been treated previously with sulfonamides. Duration of therapy varied from one to twelve days. The most striking results were obtained in those patients treated during the first attack of illness and in those patients who received a combination of oral and intramuscular streptomycin. The symptomatic improvement was associated with disappearance of the dysentery bacilli from the stools. No relapses were recorded in this series of patients.

Two patients with acute gastroenteritis and blood cultures positive for Salmonella organisms were well at the end of seven days' treatment with streptomycin given parenterally and orally.

Oral streptomycin (100 mg. per kilogram daily for four to seven days) treatment of three infants with Salmonella diarrheas resulted in remission of symptoms and in cultures negative for Salmonella for a three-week follow-up period. Of five patients with recurrent diarrhea showing Salmonella organisms, two received oral streptomycin without effect. One of these and three others of this group when treated with combined oral and intramuscular streptomycin obtained symptomatic relief and cultures remained negative for Salmonella species. In follow-up, these patients had mild abdominal cramps and loose stools, but Salmonella did not reappear.

Colitis:[11] Of the patients with nonspecific ulcerative colitis, nine of those in the acute active phase with systemic manifestations had remissions of symptoms while receiving combined oral and parenteral streptomycin therapy, but the results were not permanent and there was no striking improvement as evidenced by sigmoidoscopic examination. The amelioration of symptoms obtained by streptomycin therapy may be valuable in restoring the patient sufficiently so that surgery can be more safely undertaken. Seven patients in the chronic static phase of colitis showed no improvement whatsoever. In our experience the use of streptomycin is not justified in the medical management of idiopathic ulcerative colitis.

Streptomycin therapy was tested in two patients with amoebic colitis and found to be of no value.

Infantile Diarrhea:[11] Thirteen infants suffering from epidemic diarrhea of unknown etiology received oral streptomycin therapy in a dosage of 0.1 Gm. per pound per day incorporated in the milk formula. At the start of treatment all were in shock and severely dehydrated. It seemed that streptomycin was the determining factor in saving at least four of the ten survivors. Vigorous fluid and protein replacement were important features of the therapeutic program.

Peritonitis: Sixty-three patients have been treated with streptomycin for peritonitis of fecal origin.[14] Fifty-eight patients recovered and five died. Three of the fatalities were instances of generalized fibrinopurulent peritonitis, and streptomycin was added to the therapy after the patients had become moribund. Eighteen of the patients who recovered were treated with streptomycin alone; thirty were treated with streptomycin and penicillin; and ten were treated with streptomycin, penicillin, and sulfadiazine. The most striking response was associated with the use of streptomycin in early spreading peritonitis, whether used alone or in combination with the other antibiotic agents. Approximately the same course was seen in the resolution of established peritoneal suppuration under streptomycin therapy as under massive doses of penicillin. The infections that had already localized showed less consistent response to streptomycin. The impression was received that more rapid resolution of these lesions resulted with combined streptomycin and penicillin therapy. The most consistently beneficial effects were pursuant to the concurrent intramuscular administration of 0.3 Gm. of streptomycin and 100,000 units of penicillin every three hours. It seems likely that the complementary antibacterial spectra of these two antibiotics will favor their combined use for peritonitis of polybacterial etiology.

Wound Infections.—This unit is especially concerned with identifying the indications for, the dosage of, and the adjuvant utility of streptomycin in the treatment of impending and established wound infections. At this writing, data on the results of therapy in sixty-one patients with infections of soft tissues have been examined critically. Although these data are insufficient to establish precise clinical standards, these cases are sufficiently representative to indicate certain possibilities and limitations of the antibiotic. Streptomycin apparently has its chief field of usefulness in the therapy of cellulitis. Particularly favorable results were obtained in acute mixed gram-positive and gram-negative infections which were not responding to, or which developed in the face of, penicillin therapy. Another use for the drug appears to be the gram-positive coccal infection which has not responded to maximal doses of penicillin within seventy-two hours, or earlier if in vitro evidence of penicillin fastness has been secured. A third indication for use is the occasional case of streptomycin-sensitive gram-positive coccal infection developing in the patient with idiosyncrasy to penicillin. It is worthy of note that the mixed infections in this series occurred predominantly below the level of the diaphragm. No strikingly beneficial results have been noted following topical application of streptomycin to wounds. It is well to recall that the mere presence of gram-negative organisms in a wound is not per se an indication for streptomycin therapy. These bacteria are a feature of necrotic tissue and are eliminated only when all such tissue is removed. The only indication for topical streptomycin therapy is in conjunction with débridement. The dosage of streptomycin when used alone has been 2 to 3 Gm. per day. When used in combination with penicillin, the dosage recommended at present is 0.25 Gm. together with 50,000 units of penicillin, given intramuscularly every four hours. Investigations to establish the optimum dosage of the combination are now being conducted.

Two hundred and fifty-eight complex wound infections of battle casualties were also evaluated. Almost all of them were associated with chronic osteitis, fibrosis, and diminished local circulation. Usually a mixed bacterial flora was present. Staphylococci and streptococci were almost constantly identified and clostridia were present in approximately one-fourth of the patients. A variety of gram-negative bacteria including proteus, coli, pseudomonas, and aerogenes were associated. Of these patients, ninety-eight were considered to be benefited and one hundred and sixty were considered as not benefited by streptomycin.

The main task of arresting chronic infection in bone is surgical, not chemotherapeutic. Penicillin and to a lesser degree, streptomycin, relieve the surgeon's anxiety about sepsis and protect against spread of infection attending traumatic surgical procedures. Chemotherapy has a definite place in the postoperative management of the bone cavity. Bacteria harbored in residual dead tissue and blood clots perpetuate infection unless eradicated. Only local chemotherapy can soak these tissues in high enough concentration. In our experience, combined penicillin and streptomycin therapy is distinctly advantageous in the management of chronic osteitis from the time of sequestrectomy through the time of diverse reconstructive procedures.

Tuberculosis.—Streptomycin has now been demonstrated to be a valuable agent in certain forms of tuberculosis. In the pulmonary forms, administration parenterally of streptomycin in daily doses of 2.0 to 3.0 Gm. a day for periods up to one hundred and twenty days results in improvement above and beyond that expected from bed rest alone in 50 per cent of the patients. The exudative phase of the infection responds most readily, while fibrotic lesions remain unchanged. Streptomycin in conjunction with collapse therapy enhances the prospects of clinical arrest of the disease in properly selected cases. This antibiotic affords protection against spread of infection postoperatively following thoracoplasty. The results in pharyngitis, laryngitis, and bronchogenic tuberculosis are generally encouraging. Streptomycin has some effect in miliary tuberculosis and in tuberculous meningitis, though relapse following withdrawal of the drug must be anticipated. Streptomycin in combination with appropriate surgical treatment seems indicated in hematogenous tuberculosis with soft tissue involvement and in bone tuberculosis, though observations are still limited.

Streptomycin therapy of tuberculosis is not without danger. There is some relationship between toxicity and dosage and duration of therapy (see Untoward Reactions). Central nervous system effects, especially on the vestibular portion of the eighth cranial nerve, are a common feature of the courses of therapy necessary to affect tuberculous lesions. These effects are slow to reverse themselves and some damage may be permanent.

UNTOWARD REACTIONS

The case records of eleven hundred and fifty-three patients in this series were reviewed and the untoward reactions were tabulated. The over-all incidence of side effects was 27.9 per cent (three hundred and twenty-two patients). In discussing these reactions from the standpoint of incidence, severity, and cause,

it is emphasized that this series was begun in 1945, when streptomycin was relatively impure. As noted by McDermott,[4] certain untoward reactions are probably the effects of impurities in the streptomycin, while others are caused by the streptomycin molecule itself. It might be pointed out that a number of the side reactions recorded here are now only of historic interest.

Neurologic Disturbances.—These are the most important. There are two types, the persistent or slowly regressing, and the transient. Of the transient reactions, circumoral pallor and tingling of the face and extremities were recorded fifty times. This reaction has appeared as early as following the first intramuscular injection. Disappearance in the face of continued therapy is usual. Flushing of the skin is an occasional concomitant. Tinnitus, which also may appear early, was noted in twenty-eight patients. Vertigo may be either transient or persistent, and its appearance should be reason for caution. Neurologic examination is indicated when this symptom appears. Transient vertigo appeared usually between the third and tenth days of treatment and lasted one or more days. It is recorded thirty times, which may be an underestimation of the incidence. Persi[illegible]ng vertigo, which was accompanied by an ataxic gait and absence of vestibular response to caloric tests, was noted in fifty-six patients (5 per cent). All were on courses of therapy ranging from twenty-one to one hundred and twenty days. The incidence was highest in these treated for one hundred twenty days. The earliest appearance of this disturbance recorded is in a patient receiving 3 Gm. of streptomycin daily for fourteen days. The incidence in eighty-one patients receiving 0.5 Gm. of streptomycin every four hours intramuscularly up to a total of 50 Gm. was 3.7 per cent.[3] This reaction is slow to reverse itself. Partial deafness was recorded in twelve patients (1 per cent); all were patients with tuberculosis on a one hundred twenty-day program.

Sensitizations.—Dermatoses occurred in thirty-five patients. These reactions were accompanied by pruritis and usually by fever and eosinophilia. The manifestations varied from localized macular eruptions over the flexion creases of the forearms or over the sites of injection to generalized rashes. In six patients urticaria presented. The usual appearance of sensitization reactions was between the fifth and tenth days of therapy. Withdrawal of the drug always resulted in subsidence. Reappearance did not always follow a second course of treatment. In some patients the reaction gradually disappeared in the face of continued streptomycin administration in conjunction with antihistamine drug therapy. There were four instances of exfoliative dermatitis, all in patients with tuberculosis. This reaction is serious and requires prompt withdrawal of the drug. Disappearance is gradual.

Urinary Phenomena.—Albumin and microscopic examinations were made on a series of forty patients receiving parenteral streptomycin therapy. Slight albuminuria occurred in all instances and cylindruria in twenty-eight. These findings were not present at the conclusion of treatment. Renal irritation in no instance was regarded as sufficiently important to interrupt therapy. Tests for azotemia should be carried out on patients with evidence of prior renal

damage or on patients whose course of therapy is extended beyond two weeks.

Histamine-like Reactions.—These are transient and relatively uncommon with presently used streptomycin. In this series, headache was recorded fifty-five times; nausea and vomiting, twenty-three times; arthralgia, thirty-four times; and asymptomatic fall of 20 mm. Hg or more of systolic blood pressure, ten times.

Local Irritations.—This complaint, formerly universal, is now less frequently encountered. In our experience, the intramuscular injections of present batches of streptomycin are still followed by some pain and soreness, more so than in the use of penicillin, but we no longer make a practice of diluting the streptomycin in procaine solution. It is our routine to rotate the sites of injection among the deltoids, the hips, and the thighs.

On the basis of this experience, it is concluded that the use of highly purified streptomycin is justified in the treatment of all types of serious infections due to susceptible bacteria. Treatment certainly can be continued with safety for ten days.

SUMMARY

1. Experiences with streptomycin therapy in twelve hundred patients with infections treated in U. S. Army Hospitals have been presented.

2. The wide variation in sensitivity of bacterial species to streptomycin emphasizes the importance of checking the causative organisms in the laboratory for their susceptibility prior to therapy wherever possible. The cardinal principles which govern in the various fields of medicine and surgery must be fulfilled in conjunction with the chemotherapeutic program. Failure of the host to kill small numbers of resistant organisms, or the presence initially of many resistant forms, will result in ineffective drug therapy in many instances and in the persistence of drug-fast bacteria. Drug-fastness to streptomycin is specific and is not carried over to other chemotherapeutic agents.

3. Present indications for the drug include gram-negative urinary tract infections, tularemia, bacteriemia, pneumonia, and meningitis due to susceptible organisms, and certain otolaryngologic conditions associated with gram-negative bacteria.

4. Bacterial pneumonitides caused by streptomycin-susceptible organisms are likely to show good responses to streptomycin therapy. Results in bronchiectasis and empyema have not been spectacular.

5. In limited experience, acute brucellosis with bacteriemia was favorably influenced when streptomycin was given in combination with sulfadiazine. The drug is of no value in "chronic" brucellosis.

6. Results in typhoid fever have not been encouraging.

7. Streptomycin shows promise in the treatment of bacillary dysentery refractory to sulfonamides and in some types of Salmonella infection. It may be influential in bringing about a remission in active idiopathic ulcerative colitis with severe secondary infection caused by susceptible organisms. The drug is not useful against amoebic colitis.

8. Striking results have been obtained with streptomycin therapy in a few patients with early diffuse peritonitis, but the most consistent beneficial

effects were noted when the streptomycin was given in combination with penicillin.

9. Streptomycin is complementary to penicillin in the surgical management of wound infections, and may be of value in certain lesions heavily infected with fecal organisms.

10. A general statement has been made relative to the preliminary results of streptomycin therapy in tuberculosis.

11. The incidence and type of untoward reactions encountered in this series have been presented. It is concluded that the toxicity of presently available streptomycin is sufficiently low to justify its use in serious infections against which it has been shown to be effective.

ADDENDUM

Seven patients in all have completed a course of therapy to date, tha[illegible] 3 Gm. of streptomycin per day together with 6 Gm. of sulfadiazine, for an average of twenty-one days. The results of therapy are as follows. Two patients are well and symptom free eighteen months following treatment. A third patient has been free of complaints for nine months except for arthralgia of both knee joints and the proximal phalanges of the third and fourth fingers of the right hand. In this patient the agglutination test for Brucella organisms was positive once in a dilution of 1:160, but negative when repeated. Opsonocytophagic test was performed by the National Institute of Health and was reported positive in 1:40 strength. This patient has been seen by a number of consultants for joint diseases who feel that the arthritic process is rheumatoid rather than due to brucellosis. In two other patients relapses occurred on the seventh and twelfth weeks after treatment. A second course was not given because both patients suffered moderately severe vestibular disturbances. A sixth patient is well at this writing, but the period of observation has been for a duration of only three months. A seventh patient, in whom positive blood cultures were never obtained, was uninfluenced by the combined therapy.

REFERENCES

1. The Committee on Therapeutics and Other Agents, National Research Council, Chester S. Keefer, Chairman: Streptomycin in the Treatment of Infections. A Report of One Thousand Cases, J. A. M. A. **132**: 4, 1946.
2. DeBakey, M. E., and Pulaski, E. J.: An Analysis of Experiences With Streptomycin in United States Army Hospitals, Preliminary Report, Surgery **20**: 749, 1946.
3. Fowler, E. P., and Seligman, E.: Otic Complications of Streptomycin Therapy, J. A. M. A. **133**: 87, 1947.
4. McDermott, W.: Toxicity of Streptomycin, Am. J. Med. **2**: 491, 1947.
5. Murray, R., Paine, T. F., and Finland, M.: Streptomycin, New England J. Med. **236**: 701, 748, 1947.
6. Pulaski, E. J., and Sprinz, H.: Streptomycin in Surgical Infections. I. Laboratory Studies, Ann. Surg. **125**: 194, 1947.
7. Pulaski, E. J.: Streptomycin in Surgical Infections. II. Infections of Genito-urinary Tract, Ann. Surg. **124**: 392, 1946.
8. Pulaski, E. J.: Streptomycin Therapy of Tularemia in United States Army Hospitals, Am. J. M. Sc. **214**: 144, 1947.
9. Pulaski, E. J.: Streptomycin Therapy of Penicillin-Resistant and Sulfonamide-Resistant Specific and Nonspecific Urethritis, J. Ven. Dis. Inform. **28**: 1, 1947.
10. Pulaski, E. J., and Amspacher, W. H.: Streptomycin Therapy for Bacteremia, Am. J. Surg. **73**: 347, 1947.
11. Pulaski, E. J., and Amspacher, W. H.: Streptomycin Therapy for Certain Infections of Intestinal Origin, New England J. Med. **237**: 419, 1947.
12. Pulaski, E. J., and Amspacher, W. H.: Streptomycin Therapy in Urinary Tract Infections, Surg., Gynec. & Obst. **85**: 107, 1947.
13. Pulaski, E. J., and Matthews, C. S.: Streptomycin in Surgical Infections. III. Otitis Externa, Otitis Media, Mastoiditis, Brain Abscess and Meningitis, Arch. Otolaryng. **45**: 503, 1947.
14. Pulaski, E. J., Seeley, S. F., and Matthews, C. S.: Streptomycin in Surgical Infections. IV. Peritonitis, Surgery **22**: 889, 1947.
15. Waksman, S. A., and Schatz, A. A.: Streptomycin Origin, Nature, Properties, J. Am. Pharm. A. (Scient. Ed.) **34**: 273, 1945.

SEROLOGIC STUDIES OF INFLUENZA MADE IN BOSTON DURING THE WINTER OF 1945-1946

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THE 1945 epidemic of influenza B which was recognized in many parts of this country and in other countries[1-13] reached its peak of incidence in and around Boston during the latter half of December, 1945. This epidemic offered an opportunity to make some serologic observations during and after an outbreak of influenza B similar to those that were made in this laboratory during and after the outbreak of influenza A that occurred here in December, 1943.[14] The reactions of the viruses obtained during the height of the 1943 epidemic and the antibody response to characteristic infections occurring at that time indicated that the epidemic cases were caused chiefly by strains of influenza A virus similar to PR8. The postepidemic cases of clinical influenza, on the other hand, were atypical not only with respect to the viruses which were isolated from some of them but also with respect to their antibody responses to both their own strains and to the standard PR8 strain. There was no evidence of infection with influenza B in any of the cases studied.

After the influenza A epidemic it was also found that the sera of most persons giving a characteristic history of influenza during the period of the epidemic had significantly elevated titers of antibodies to PR8 demonstrable for as long as twelve weeks after the onset of these symptoms. Such elevated titers could not be found during the same period in the sera of persons who denied having had any symptoms of acute respiratory infections or who had had symptoms which could be distinguished readily as those of the common cold and not influenza.

Of particular interest were the high titers of antibodies to PR8 that were demonstrated in severe cases of bacterial pneumonia which occurred during and shortly after the epidemic and in which there was an antecedent history of clinical influenza. Those findings and the isolation of influenza virus from the lungs of three patients with fatal cases suggested that the occurrence and severity of the pneumonia in such cases were related to the influenza virus infection.

The present paper deals chiefly with the results of serologic tests for influenza antibodies in cases of clinical influenza and other acute respiratory infections which occurred during and for a few weeks after the epidemic prevalence of influenza B. The results of tests made on two other groups of sera are also included: one group consisted of paired sera obtained during and shortly after

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the height of the epidemic from persons who were entirely free of illness, and the other consisted of single samples from a somewhat larger group of persons obtained a few weeks after the epidemic had subsided. The results of attempts at isolation and identification of virus from patients with acute cases in this epidemic and the serologic findings in cases of pneumonia are left for separate consideration elsewhere.[15, 16]

MATERIALS AND METHODS

Serologic Tests.—Both inhibition of chicken cell agglutination and complement fixation tests were carried out in this study. The antigens employed included the PR8 strain* of influenza A and the Lee strain* of influenza B and two strains, WC and MF, of influenza B isolated from patients early in the course of this study. The details of the collection and preservation of the sera, the serologic methods, and the manner of reading and recording the end points were identical with those used in a collateral study.[12]

Cases Studied.—Included in the present studies were members of the hospital and laboratory staff, both with and without illness, and patients admitted to the regular adult medical wards of the hospital between the middle of December, 1945, and early in March, 1946. Control sera obtained early in the study were available from some of the hospital and laboratory personnel who subsequently developed respiratory infections. With a few exceptions (noted in Table III) patients admitted to the hospital with clinical and x-ray findings of pneumonia are omitted here, since they are considered separately elsewhere.[16]

Clinical Findings.—The clinical features of the cases of influenza were quite characteristic and were similar to those observed in the Needham outbreak.[12] In the patients who were seen during the acute illness, an attempt was made to evaluate and classify the clinical findings at that time. This was done again in all of the patients when all of the clinical findings were available and entirely without reference to the serologic data. Those with findings that fit the characteristic picture of the epidemic disease, namely chills or chilly sensations, fever, prostration, headache, eyeball soreness, generalized aches, mild sore throat, and some cough, were classified as having clinical influenza and the severity of their illness was graded 1 plus, 2 plus, or 3 plus. Patients with a minimum of fever and systemic symptoms but with moderate or marked coryza, with or without cough, were classified for convenience as having "colds." A third type of illness was also recognized in which the systemic symptoms resembled somewhat those of influenza but the patient suffered chiefly from anorexia, nausea, vomiting, and diarrhea and had no symptoms referable to the respiratory tract —so-called "intestinal flu."

RESULTS

The cases studied have been arranged for convenience into two main groups, one including patients with acute respiratory infections and the other those without such infections. The former was further arranged into four subgroups to include: patients in whom there was serologic evidence of infection with

*Originally obtained from Dr. Thomas Francis, Jr., Ann Arbor, Mich.

influenza B; patients in whom studies of acute and convalescent phase sera failed to yield evidence of infection with either influenza A or B; patients with severe acute respiratory infection, including five who had fatal cases, from whom sera were available only during the acute phase of the illness; and finally, those in whom there was serologic evidence of infection with influenza A. The group without respiratory illness was composed of two subgroups: a small number of hospital and laboratory workers from whom control sera were obtained in the second week of December and again about three weeks later and in whom no respiratory illness developed during the course of this study; and hospital personnel and ward patients who were under treatment at the time for nonfebrile illness other than acute respiratory infections and who each contributed a single specimen of serum toward the end of this study. The results of the serologic findings in each of these groups of cases will be considered separately. They will then be summarized in order to bring the findings in the various groups into proper perspective.

PATIENTS WITH ACUTE RESPIRATORY INFECTIONS.—

Group I. Patients With Serologic Evidence of Influenza B Infection.—The relevant findings in twenty-three such patients are listed in Table I. In each of these patients there was at least a fourfold rise in titer of the influenza B antibodies, and much greater rises were noted in almost every instance. For the most part, rises of similar grade were demonstrated by both the agglutinin inhibition and the complement fixation tests with the Lee strain and with the two epidemic strains. There were very few discrepancies, as for example in Patients 4, 7, and 9 in whom the agglutinin inhibition test with one or another of the B viruses showed little or no rise while significant rises were noted by complement fixation with the same virus or, indeed, by the same test with another of the B strains.

There was no appreciable rise in titer of PR8 antibodies demonstrable by either of the tests used in any of these cases. The initial titers of these antibodies were low except for a few elevated titers of agglutinin inhibition.

Each of these patients, with the exception of the first one, had an infection which was considered to be characteristic of the epidemic influenza and which began between Dec. 12 and Jan. 3. The illness, however, varied considerably in these cases, both with respect to the height and duration of the fever and the severity of the systemic symptoms. Leucocyte counts were done during the acute illness in most of these cases. Only two patients showed elevated counts, 11,700 in one and 14,000 in the other, and both of these patients had moderately severe tracheobronchitis without physical or x-ray signs of pulmonary infiltration. The leucocyte counts in slightly more than half of the remaining cases ranged between 4,200 and 6,500 and in the others they ranged between 7,000 and 10,000.

The only patient without the characteristic findings of clinical influenza (Patient 1) had a mild coryza which began Dec. 6, lasted for ten days, and was not accompanied by fever or systemic symptoms. In this case a rise in the B antibodies occurred from low titers after the eighth day. In five additional

TABLE I. SEROLOGIC FINDINGS IN PATIENTS WITH EVIDENCE OF INFLUENZA B INFECTION

PATIENT	CLINICAL INFLUENZA		AVERAGE TITER OF INFLUENZA ANTIBODIES									
				PR8		LEE		WC		MF	
	DATE OF ONSET	SEVERITY	DATE OF SERUM	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION
1	12/ 6	C	12/14	13	6	4	4	6	10	8	10
			1/ 2	13	6	10	25	12	40	32	40
2	12/12	+++	12/15	8	6	5	3	2	2		
			12/27	6	6	12	56	46	64		
3	12/13	++	12/14	24	2	15	3	3†			
			1/ 2	24	2	56	44	24			
4	12/13	+	12/14	15	7	12	6	16	7		
			1/ 2	15	9	32	16	16	32		
5	12/14	+	12/14	24	2	11	4	2	3	5†	6
			12/27	27	3	115	272	40	180	64	192
6	12/15	+	12/26	16	10	192	56				
	12/23*	++	1/ 9	16	7	512	384				
7	12/16	++	1/20	32	9	5	2	2	2		
			1/26	18	12	10	52	12	64		
8	12/17	+++	12/17	32	7	27	9	16	7	8	6
			1/ 5	32	8	278	145	256	128	128	96
9	12/18	+	12/18	2	5	2	2	2	2		
			1/ 4	2	3	4	28	8	28		
10	12/18	+++	12/21	20	32	2	2	2	2	3	2
			12/28	20	32	1024	192	48	256	96	192
11	12/ 8	C	12/23	256	32	6	5	2	7	3	7
	12/20	++	12/26	128	28	64	192	160	320	64	256
12	11/20	C	12/21	20	8	21	6	8	5	5	5
	12/21	++	1/ 8	20	7	117	28	64	24	32	16
13	12/21	++	12/23	64	10	14	4	4	3	3	3
			1/16	64	9	59	13	32	12	12	10
14	12/24	++	12/29	8	12	5	2	2	2	2	2
			1/ 2	6	8	21	28	16	32	12	12
15	12/17	D	12/26	32	24	4	2	2	2	2	2
	12/25	++	1/ 4	24	32	192	104	64	96	64	56
16	12/26	++	12/28	16	24	4	2	2	2	4	2
			1/ 6	16	40	36	480	256	896	256	896
17	12/27	++	1/ 2	3	3	3	5	2	8	2	6
			1/8	10	8	132	112	48	128	96	128
18	12/27	+++	1/ 3	8	3	6	24				
			1/10	8	2	24	192				
19	12/27	C	1/ 2	27	9	5	9	4	12		
	12/30	++	1/10	51	16	64	44	32	56		
20	12/30	++	12/31	6	3	6	2	3	2	4	2
			1/ 8	3	5	64	165	64	224	64	128
21	12/ 4	C	1/ 3	64	24	8	4				
	1/ 1	+++	1/10	48	20	320	512				
22	1/ 1	+++	1/ 5	8	4	32	14				
			1/11	8	3	2048	896				
23	1/ 3	++	1/ 4	128	19	32	7				
			1/22	96	26	192	96				

Only two sera are listed in each case. Intermediate and later sera were obtained and studied in almost every case, but these are omitted from this table and from Tables II and III. Those selected for inclusion in these tables showed the maximum changes observed.

Symptoms considered typical of influenza are graded according to severity as +, ++, or +++; C, coryza and/or cough without systemic symptoms; D, gastrointestinal symptoms predominant.

*Relapse with increase in severity.

†Homologous virus.

patients there was another distinct illness preceding the one considered to be influenza. This antecedent illness was classified as a common cold in four instances and as "intestinal flu" in the fifth. The interval between the onset of the two illnesses was three days in one patient and from eight to thirty-one days in the others, and the initial serum was obtained up to three days after the onset of the second illness. In none of these cases, however, was a significantly elevated titer of antibodies for either the A or B viruses demonstrated in the acute phase serum to suggest a possible earlier influenzal infection; Patient 11 may be an exception since an elevated titer of agglutinin inhibition was demonstrated in the first blood with the PR8 strain. The corresponding complement fixation titer, however, was not correspondingly elevated in that serum, and the interpretation of this finding must remain in doubt. Patient 6 was admitted on the eleventh day after the onset of what was considered to be a typical but mild influenza from which he seemed to improve until a relapse occurred with similar but somewhat more severe symptoms three days prior to entry. The Lee antibodies were elevated in the initial blood obtained on entry but later showed a significant rise, suggesting that the initial infection was indeed influenza B.

Group II. Patients Without Evidence of Influenza A or B Infection.—For reasons which will became apparent later, the patients in whom tests of acute and convalescent sera failed to disclose any evidence of infection with either influenza A or B were divided into two groups according to the date of onset of their illness. Those in whom the illness began before the second week in January, corresponding to the dates of onset in the cases just described, will be considered first. The few patients whose illness began after the middle of January will then be discussed.

A. Patients With Onset of Illness Prior to Mid-January Whose Sera Failed to Show an Antibody Rise: There were twenty-one such patients and the findings are shown in the first part of Table II (Patients 24 to 44). There was no antibody rise whatever in any of these cases to PR8, Lee B, or either of the epidemic strains demonstrable by either of the tests used. The initial titers in these cases were low, for the most part, and corresponded to those observed in the patients in whom rises in influenza B antibodies were demonstrated.

The illness in thirteen of these twenty-one patients was similar in most respects to that observed in the previous group and was classified as influenza of varying severity. In the other eight patients the illness clinically either resembled the common cold (four cases) or the symptoms were predominantly gastrointestinal (two cases) or both of these types of illness occurred in succession (two cases).

B. Patients Whose Illness Began After Mid-January and Whose Sera Failed to Show an Antibody Rise: There were only eight patients in this group and the findings are shown in the lower part of Table II (Patients 45 to 52). In this small group there seemed to be a higher proportion of sera in which agglutinin-inhibiting antibodies for PR8 were in the high range. The titers otherwise were similar to those observed prior to Jan. 7. Each of the patients

TABLE II. PATIENTS WITH ACUTE RESPIRATORY INFECTIONS LACKING SEROLOGIC EVIDENCE OF INFECTION WITH INFLUENZA A OR B

PATIENT	CLINICAL INFLUENZA		AVERAGE TITER OF INFLUENZA ANTIBODIES									
				PR8		LEE		WC		MF	
	DATE OF ONSET	SEVERITY	DATE OF SERUM	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION
24	11/25	C	12/14	80	48	44	13				
			12/31	80	48	60	13				
25	12/ 2	C	12/14	8	12	4	12				
			12/31	8	5	6	12				
26	12/ 7	C	12/20	32	20	24	12				
			12/24	32	24	32	12				
27	11/26	D	12/14	6	20	24	12				
	12/ 9	C	1/ 2	6	10	8	10				
28	12/11	+ +	12/17	13	13	19	6			6	6
			1/ 3	10	6	15	5			8	6
29	12/11	+ +	12/14	16	3	10	9	12	12	12	12
			1/ 5	16	4	10	10	12	14	12	12
30	12/11	+ +	12/16	4	7	4	7	12	4	3	5
			12/31	4	3	3	3	12	4	4	6
31	12/11	D	12/18	28	5	12	3				
			1/ 4	28	5	3	12				
32	12/12	+ +	12/12	96	80	72	16	3	10	3	12
			1/ 2	96	36	88	20	4	8	3	12
33	12/13	D	12/14	88	10	24	15	12	10	5	6
	12/14	C	12/28	48	20	34	27	5	24	5	9
34	12/14	C	12/17	12	12	12	12				
			1/ 2	12	12	12	12				
35	12/14	+	12/14	16	28	12	36	16	40	16	28
			1/ 2	16	28	14	32	16	40	16	32
36	12/18	D	12/18	16	6	5	3	12	3	6	3
			1/ 4	16	6	4	3	12	3	12	3
37	12/19	+	12/13	12	5	7	12	4	20	4	16
			1/ 2	12	10	9	8	3	12	4	14
38	12/19	+ +	12/18	16	23	12	32	24	24	6	20
			1/ 2	16	36	3	40	24	28	6	24
39	12/30	+ + +	12/28	16	24	6	6	12	6	6	8
			1/ 7	32	24	3	5	12	5	6	10
40	12/22	+	12/24	6	24	3	4	4	12	12	3
	1/ 4		1/11	6	16	3	4	3	12	12	5
41	12/23	+	12/14	8	5	14	6	4	10	24	10
			1/ 4	6	7	14	7	4	10	16	10
42	12/23	+ +	1/12	64	12	24	12				
			1/18	64	12	32	12				
43	1/ 3	+ +	1/ 3	80	28	72	24				
			1/15	80	28	72	24				
44	1/ 6	+	1/ 2	20	16	64	32				
			1/17	20	16	96	32				
45	1/18	+	2/ 4	64	3	12	12				
			2/14	128	6	12	12				
46	1/21	+ +	2/ 3	64	16	24	4				
			2/21	96	11	8	3				
47	2/18	+ +	2/20	112	20	28	4				
			3 /5	171	32	27	4				
48	1/17	+ +	2/21	64	6	64	14				
	2/20	C	3/12	64	6	64	20				
49	3/ 4	+ + +	2/26	25	7	36	8				
			3/11	24	7	36	7				
50	3/ 6	+ + +	3/ 7	16	20	3	14				
			4/ 1	24	20	3	12				
51	3/12	+ +	3/13	192	28	32	8				
			4/ 2	192	28	24	7				
52	3/12	+ +	3/13	64	28	32	20				
			3/28	64	32	32	16				

Symptoms considered typical of influenza are graded according to severity as +, + +, or + + +; C, coryza and/or cough without systemic symptoms; D, gastrointestinal symptoms predominant.

had an illness which was characteristic of the epidemic influenza. One had a second illness classified as a cold which began one day prior to the time the initial blood was taken.

Group III. Findings in Acute Phase Sera.—There was a group of twenty patients with severe acute respiratory infections from whom sera were obtained during the acute phase of the illness, but later specimens were not available. Almost all of them had symptoms that began before mid-January and were classified as those of influenza. In some of them, these symptoms were followed by the typical findings of pneumococcal pneumonia. There were five deaths in this group but autopsies were not obtained in any of them. Agglutinin inhibition titers greater than 64 against PR8 were noted in two patients and against Lee in one patient, but the corresponding complement fixation titers were low in each instance. The remaining titers of antibodies to the PR8 and Lee strains in this group of cases were all low and corresponded to those found in the acute phase sera in those patients who later showed a rise against the B strains.

Group IV. Patients With Serologic Evidence of Influenza A Infection.—There were thirteen patients in whom the serologic findings indicated infection with influenza A. The relevant data in these cases are summarized in Table III. The onset of illness in all of these cases occurred between Jan. 18 and Feb. 3, except in Patient 53 whose symptoms began Dec. 28. In five of these patients (Patients 56, 57, 62, 63, and 64) the initial serum obtained between the seventh and twenty-first day after the onset already had significantly elevated PR8 antibody titers. However, in contrast to the isolated instances of elevated titers of agglutinin inhibition by PR8 noted in the previous groups of cases in which there was no corresponding elevation in the titer of complement-fixing antibodies obtained with the same virus, the elevated titers in the present five patients were demonstrated by both tests. In the remaining eight patients, significant and usually marked rises in antibodies occurred to the PR8 but not to the Lee virus.

Several attempts to isolate a virus from pharyngeal washings obtained during the first day of illness in two of the patients (Patients 54 and 60) were entirely unsuccessful.

There were two patients in this group in whom there was definite serologic evidence of successive infections, first with influenza B and then with influenza A. One of these, Patient 58, was originally admitted to the hospital for influenza which began Nov. 30 and was accompanied by characteristic symptoms and physical and x-ray findings of atypical pneumonia. The patient recovered fully and later was readmitted because of a second attack of influenza, the symptoms of which began Jan. 27. The titer of antibodies for the Lee strain had risen from 20 to 640 by agglutinin inhibition and from 3 to 1,792 by complement fixation during the first illness. These titers had already dropped appreciably at the time of the second hospital admission. There had been no rise in PR8 antibodies during the first attack.

The early symptoms in the original attack of influenza in the second patient, Patient 59, were quite characteristic of the epidemic disease and began on Dec. 24. These were followed by a typical course of type 5 pneumococcal pneumonia which began abruptly on Dec. 29. During this illness there was a marked rise

TABLE III. PATIENTS WITH EVIDENCE OF INFLUENZA A INFECTION

PATIENT	CLINICAL INFLUENZA		AVERAGE TITER OF INFLUENZA ANTIBODIES				
				PR8		LEE	
	DATE OF ONSET	SEVERITY	DATE OF SERUM	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION
53	12/28	C	12/31	32	4	12	6
			1/11	256	96	12	5
54	1/18	+ + +	1/19	12	20	64	44
			1/29	36	60	56	44
55	1/21	+ +	1/ 4	28	5	3	2
			1/28	96	14	6	2
			2/26	160	28	8	2
56	1/24	+ +	2/ 4	256	160	60	16
			3/ 1	256	125	60	9
57	1/25	+*	2/15	1024	112	32	4
58	1/27	+ +†	1/28	64	22	149	168
			2/14	256	112	144	104
59	?	0‡	1/16	32	2	1280	160
			2/27	192	64	32	20
60	1/30	+ +	1/31	12	2	6	10
			2/15	160	48	8	11
61	2/ 1	+ +	2/ 7	64	24	3	14
			2/14	2048	768	3	10
62	2/ 1	+ +	2/ 8	96	144	48	2
63	2/ 1	+ + +§	2/ 8	384	72	12	4
64	2/ 2	+‖	2/18	192	100	32	2
65	2/ 3	+ +	11/31	80	48	60	13
			2/26	352	104	32	11

Symptoms considered typical of influenza are graded according to severity as +, ++, or + + +; C, coryza and/or cough without systemic symptoms; O, no symptoms.

*Bronchopneumonia; onset, 2/12; W. B. C., 11,900; etiology,?

†Had influenza with atypical pneumonia (onset, 11/30) with rise in influenza B antibodies from 20 to 640 by agglutinin inhibition test and from 3 to 1,792 by complement fixation test. Cold agglutinins could not be demonstrated in any of the sera.

‡Influenza (onset, 12/24) and type 5 pneumococcal bronchopneumonia (onset, 12/29), with marked rise in influenza B antibodies (from titer of 4 in acute phase). No definite symptoms associated with influenza A infection.

§Type 5 pneumococcal bronchopneumonia; onset 2/6; W. B. C., 6,600.

‖Type 4 pneumococcal lobar pneumonia; onset 2/2; W. B. C., 3,000.

in antibodies to PR8 but not to the Lee strain although the initial titers to both of these viruses were low. The patient was discharged from the hospital and subsequent specimens of serum were obtained when the patient returned on two occasions for checkup examinations. The sharp rise in PR8 antibodies that occurred following this patient's discharge was not associated with any subjective or objective evidence of infection.

In all but one of the remaining patients the infections which occasioned the rise in influenza A antibodies could not be distinguished clinically from those which were associated with the rises in influenza B antibodies. In Patient 53 the illness was characterized by coryza, slight sore throat, and slight productive cough without fever or systemic symptoms; the leucocyte count was 8,700.

PATIENTS WITHOUT ACUTE RESPIRATORY INFECTIONS.—

Group V. Serologic Findings During the Influenza B Epidemic in Persons Without Respiratory Infections.—There was a group of eleven hospital and

TABLE IV. RESULTS OF TESTS FOR INFLUENZA ANTIBODIES IN SERUMS OBTAINED BETWEEN FEBRUARY 16 AND 27, 1946, FROM HOSPITAL PATIENTS AND NORMAL PERSONS WITHOUT ACUTE RESPIRATORY INFECTION

PATIENT	RECENT HISTORY OF CLINICAL INFLUENZA*	DATE OF SERUM	AVERAGE TITER OF INFLUENZA ANTIBODIES							
			PR8		LEE		WC		MF	
			AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION
66	0	2/16	32	10	16	8	6	20	4	20
67	C2	2/16	64	2	6	6	2	12	6	7
68	C1	2/16	256	12	4	2	6	20	6	20
69	C1, 2	2/16	64	6	64	20	32	40	32	20
70	C1	2/16	96	12	128	32	48	48	64	40
71	0	2/17	128	12	64	16	4	12	4	6
72	0	2/17	96	6	8	3	24	7	2	6
73	C2	2/18	96	40	64	16	192	40	8	28
74	0	2/18	256	32	12	7	8	12	8	12
75	C1	2/18	48	4	16	8	8	14	4	12
76	C1	2/18	32	5	48	7	8	20	8	20
77	0	2/18	64	8	48	12	16	20	16	28
78	0	2/18	192	24	32	6	4	3	4	5
79	C1	2/18	48	16	24	20	8	28	6	32
80	C1	2/20	64	28	96	40	32	48	64	48
81	V	2/20	512	40	512	80	128	112	192	128
82	C1	2/20	12	3	32	2	8	7	16	10
83	C2	2/20	32	20	256	24	8	24	8	112
84	C1	2/20	128	2	64	16	8	40	16	32
85	C1	2/20	96	24	64	16	32	24	32	20
86	0	2/20	32	3	24	3	2	7	2	10
87	C1	2/20	64	16	128	12	16	29	16	20
88	C1	2/20	128	14	96	12				
89	C1	2/20	28	10	128	48				
90	C1, 2	2/21	80	10	128	22				
91	C1, 2	2/21	96	20	96	16				
92	C1, 2	2/21	48	12	40	16				
93	0	2/21	8	20	8	20				
94	C2	2/21	80	20	7	20				
95	C1, 2	2/21	80	14	8	2				
96	0	2/21	80	24	12	10				
97	C1	2/21	96	20	4	2				
98	F1	2/21	48	20	96	128				
99	0	2/21	36	40	96	128				
100	C2	2/22	10	20	32	96				
101	F2	2/22	284	56	96	48				
102	C2	2/22	144	20	24	10				
103	F1	2/23	112	5	18	8				
104	0	2/23	384	48	96	40				
105	C1, F2	2/23	160	12	72	28				
106	0	2/23	24	6	10	2				
107	C2	2/23	30	6	14	3				
108	0	2/23	40	20	64	32				
109	F1	2/23	304	28	96	64				
110	0	2/23	40	5	24	12				
111	C1, 2	2/25	192	56	80	28				
112	0	2/25	96	20	14	8				
113	V	2/25	144	14	128	48				
114	C1, F1	2/25	384	32	112	32				

(Continued on page 24.)

TABLE IV—CONT'D

PATIENT	RECENT HISTORY OF CLINICAL INFLUENZA*	DATE OF SERUM	AVERAGE TITER OF INFLUENZA ANTIBODIES							
			PR8		LEE		WC		MF	
			AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION
115	V, F1	2/25	256	6	96	24				
116	0	2/26	160	12	40	8				
117	0	2/27	40	6	16	12				
118	0	2/27	128	14	9	5				
119	0	2/27	40	7	56	16				
120	F1	2/27	240	16	15	14				
121	F1	2/27	28	6	48	16				
122	F2	2/27	144	12	7	2				
123	0	2/27	48	3	5	2				
124	0	2/27	12	2	10	8				
125	0	2/27	56	10	48	14				
126	0	2/27	14	3	80	14				
127	0	2/27	20	7	16	8				

*C, History of coryza and/or cough with minimum of fever and systemic symptoms; F, symptoms suggesting clinical influenza; 1, onset between Nov. 15, 1945, and Jan. 15, 1946; 2, onset after Jan. 16, 1946; V, influenza A and B vaccine in November, 1945; O, no symptoms.

laboratory workers from whom sera were obtained during the end of the second week in December and again during or after the first week in January. They had no symptoms of any illness during this interval or in the preceding few weeks. None of these individuals showed any rise in titer of antibodies for either the PR8 or the Lee virus. Low titers were obtained in all of these sera except for agglutinin inhibition titers greater than 64 obtained in both sera from one of this group with the Lee strain without correspondingly elevated complement fixation titers.

Group VI. Influenza Antibodies in Sera Obtained After the Epidemic.—In order to obtain some idea as to whether a retrospective history of respiratory infection during the epidemic might be reflected frequently in a high influenza antibody titer a few weeks later, single specimens of sera were obtained between Feb. 16 and 23, 1946, from sixty-two individuals not included in any of the previous groups. Most of them were from young adult patients hospitalized for illnesses other than acute respiratory infections and others were from members of the hospital staff. Each person was questioned in detail concerning any illness that occurred during the preceding three months. All respiratory illnesses were then classified according to whether they most resembled colds or clinical influenza and whether they occurred before or after the middle of January. The occurrence of such infections as determined in this manner and the results of the tests for influenza antibody in their sera are listed in Table IV.

The outstanding finding in this group is the comparatively large proportion of sera showing elevated titers (greater than 64) of agglutinin inhibition of the PR8 strain without corresponding elevation of the complement fixation titers with the same strain. There were a few instances of elevated titers of agglutinin

inhibition of the Lee strain and in some of these sera the corresponding complement fixation titers were also higher than average. When this group is subdivided according to the recent history of respiratory infections, the numbers in each category are too small to permit any significant comparisons. However, except for the somewhat greater frequency of high agglutinin inhibition titers with both the PR8 and Lee strains in the person giving a recent history of clinical influenza, there seemed to be no striking predominance of significantly elevated titers in any group.

There were three persons in this group (Patients 81, 113, and 115) who had received an immunizing injection of influenza A and B vaccine in November, 1945. One of them showed elevated titers by both tests against all the four strains of virus used. The other two showed elevated agglutinin inhibition titers with the PR8 and Lee strains without correspondingly elevated complement fixation titers. One of the latter also gave a history of clinical influenza during the height of prevalence of influenza B.

Summary of the Serologic Findings.—The distribution of titers of PR8 and Lee antibodies in the seven groups that have been discussed is shown in Table V. The average titers for each of these groups are also shown. Groups I and IV* stand out clearly from among the others. In Group I there was a similar distribution of titers in the lower range of both agglutinin inhibition and complement fixation of the PR8 strain in both acute and convalescent phase sera, whereas there was a shift in Lee antibodies from low titers in the acute phase to titers in the higher range in the convalescent phase sera. In accord with this distribution the average titers were essentially the same for acute and convalescent sera in the tests done with PR8 (though the actual numbers were higher for the average agglutinin inhibition titers than for the complement fixation titers), while the average anti-Lee titers were about twenty fold greater during convalescence in the agglutinin inhibition tests and about thirty-five fold greater in the complement fixation tests.

The reverse was true in Group IV. The number of cases in this group was small, but the distribution of anti-Lee antibodies was in the low range and the average titers were low and were the same by both tests in the acute and convalescent phase sera. The tests with PR8, on the other hand, gave low titers in the acute sera and high titers during convalescence. The average anti-PR8 titers were about ten times greater in the convalescent phase sera than in the acute phase sera done with either test.

In Groups IIA, III, and V the distribution of titers and the average titers were all similar to those found in the acute phase sera in Groups I and IV. In Groups IIA and V this was true for both the early and the late sera.

Groups IIB and VI are similar in several respects and stand out from the other groups. The sera in both of these groups were obtained after the date of the last proved case of influenza B. In each instance there was a smaller proportion of very low titers and more of the intermediate and the moderately elevated titers of agglutinin inhibition with PR8. The averages of these titers

*The group designations correspond to those used in Table V and also to those used in the previous section headings in the text.

TABLE V. SUMMARY OF RESULTS OF SEROLOGIC TESTS WITH PR8 AND LEE STRAINS IN SEVERAL GROUPS OF PATIENTS

	GROUP*	STRAIN	TEST†	NUMBER OF CASES	TITER‡										
					4	8	16	32	64	128	256	512	1024	2048	AVERAGE§
I	Acute	PR8	AI	23	2	5	4	8	2	1	1				36
	Convalescent	PR8	AI	23	2	4	5	7	3	2					28
IIA	Acute	PR8	AI	21	2	4	8	2	1	4					30
	Convalescent	PR8	AI	21	2	4	6	4	2	3					29
IIB	Acute	PR8	AI	8	0	0	1	1	4	1	1				75
	Convalescent	PR8	AI	8	0	0	0	2	2	2	2				106
III	Acute	PR8	AI	20	3	4	3	6	2	1	1				36
IV	Acute	PR8	AI	8	0	0	2	3	2	1					41
	Convalescent	PR8	AI	13	0	0	0	0	1	1	7	2	1	1	416
V	Early	PR8	AI	11	3	4	2	2							13
	Late	PR8	AI	11	4	2	2	3							13
VI		PR8	AI	62	0	1	4	9	16	16	11	5			109
I	Acute	Lee	AI	23	6	8	4	4	0	0	1‖				18(11)
	Convalescent	Lee	AI	23	1	0	3	3	6	2	3	3	1	1	234
IIA	Acute	Lee	AI	21	6	3	3	5	2	2					21
	Convalescent	Lee	AI	21	7	2	5	2	3	2					23
IIB	Acute	Lee	AI	8	1	0	1	4	2						26
	Convalescent	Lee	AI	8	1	1	1	3	2						26
III	Acute	Lee	AI	20	3	2	4	3	7	1					32
IV	Acute	Lee	AI	8	2	1	1	0	2	1¶	0	0	1¶		196(23)
	Convalescent	Lee	AI	13	1	2	2	4	3	0	1				37(28)
V	Early	Lee	AI	11	3	5	1	0	1	1					20
	Late	Lee	AI	11	3	4	1	1	1	1					22
VI		Lee	AI	62	2	7	12	8	13	18	1	1			63

I	Acute	PR8	CF	23	6	6	5	6						11
	Convalescent	PR8	CF	23	5	8	4	5	1					13
IIA	Acute	PR8	CF	21	5	4	3	7	1	1				15
	Convalescent	PR8	CF	21	4	5	4	5	2	1				19
IIB	Acute	PR8	CF	8	1	2	1	4						17
	Convalescent	PR8	CF	8	0	3	1	4						16
III	Acute	PR8	CF	20	6	6	7	1						8
IV	Acute	PR8	CF	8	3	1	0	3	1					16
	Convalescent	PR8	CF	13	0	0	0	1	3	6	2	0	1	144
V	Early	PR8	CF	11	3	2	3	3						13
	Late	PR8	CF	11	2	3	5	1						11
VI		PR8	CF	62	8	13	18	17	6					16
I	Acute	Lee	CF	23	13	5	3	1	1‖					8(5)
	Convalescent	Lee	CF	23	0	0	2	4	4	3	5	4	1	177
IIA	Acute	Lee	CF	21	7	4	6	3	1					12
	Convalescent	Lee	CF	21	8	4	3	5	1					12
IIB	Acute	Lee	CF	8	3	2	2	1						9
	Convalescent	Lee	CF	8	3	2	2	1						9
III	Acute	Lee	CF	20	8	2	5	3	1	1				15
IV	Acute	Lee	CF	8	1	1	3	0	1	0	2¶			52(15)
	Convalescent	Lee	CF	13	5	1	4	1	1	1				18(10)
V	Early	Lee	CF	11	3	2	3	3						14
	Late	Lee	CF	11	3	2	4	2						12
VI		Lee	CF	62	10	12	18	12	6	4				23

*I, Patients with evidence of influenza B (Table I); II, A, cases of acute respiratory infection occurring prior to mid-January without evidence of influenza A or B (Table II, Patients 24 to 44); II, B, cases similar to those in II, A but occurring after mid-January (Table II, Patients 45-52); III, cases of acute respiratory infection in which convalescent sera were not available (including five fatal cases; IV, patients with evidence of influenza A (Table III); V, control subjects without infection; early sera obtained in mid-December, late sera in January; VI, hospital personnel and patients; single sera obtained February 16-23 (Table IV).

†AI, Inhibition of chicken cell agglutination; CF, complement fixation.

‡Each category includes intermediate titers up to next preceding one (e.g. 4 includes 4 or less; 8 includes 5-8 etc.)

§Corrected average (after excluding sera obtained on or after the seventh day or those indicated by ¶) shown in parentheses.

‖Acute blood on eleventh day.

¶Earlier influenza B infection; the values in these two cases are excluded in the corrected averages.

were two to three times greater than in all groups except Group IV (convalescent sera from the patients with influenza A). The values for the convalescent phase sera in Group IIB were slightly but not significantly higher than for the acute phase sera. On the other hand, the distribution of the complement fixation titers was the same, and the average of these titers obtained with the same virus in these two groups of cases was not elevated and corresponded to those observed in all other groups except in the convalescent phase sera in Group IV.

With respect to influenza B antibody, however, Groups IIB and VI differed. In the former, the distribution of titers was in the low range and the average titers in both the acute and convalescent phases were low and corresponded to those observed in Groups IIA, III, and V and in the acute phase sera in Group I. In Group VI, on the other hand, there was a greater proportion of intermediate and slightly elevated titers of Lee antibodies and the averages of these titers were two to three times those found in the remaining groups other than Group I (convalescent sera from patients with influenza B). In these respects the findings with the agglutinin inhibition and complement fixation tests were similar.

Comparison of Results Obtained With the Lee Strain and With Two Epidemic Strains.—It will be seen from Tables I, II, and IV that, although there were some discrepancies in individual sera, the results obtained with the standard Lee strain and with WC and MF (the two strains of influenza B virus that were isolated early in the course of the epidemic) were very similar. The average titers of all the sera of these three groups that were tested with the three strains are shown in Table VI. Considering the rather small numbers involved, the average titers obtained in each group with the three strains were remarkably similar.

TABLE VI. COMPARISON OF AVERAGE TITERS OBTAINED WITH LEE STRAIN AND WITH TWO EPIDEMIC STRAINS IN THREE GROUPS OF PATIENTS

		LEE			WC			MF		
GROUP*		NUMBER OF CASES	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	NUMBER OF CASES	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	NUMBER OF CASES	AGGLUTININ INHIBITION	COMPLEMENT FIXATION
I	Acute	22	11	5	18	4	5	12	4	4
	Convalescent	23	234	177	18	67	151	12	77	169
IIA	Acute	21	21	12	11	6	13	12	7	11
	Convalescent	21	23	12	11	6	14	12	7	12
VI		62	63	23	22	27	26	22	24	29

*See footnote *, Table V.

COMMENT

The serologic findings in Groups I and IV indicate quite clearly that both influenza B and influenza A occurred in Boston during the winter of 1945-1946. The dates of onset of illness in the patients of those two groups indicate that, for the most part, there were distinct and consecutive outbreaks. In this study

the proved cases of influenza B, with one exception, had an onset between Dec. 12 and Jan. 3; the onset in the cases of serologically proved influenza A, again with one exception, occurred between Jan. 18 and Feb. 3. Viruses giving characteristic reactions of influenza B were isolated from patients with acute cases during the former period,[15] but attempts to isolate viruses from two others with acute cases of influenza A were not successful.

The occurrence of both influenza B and A, with a predominance of the former, was also reported in the corresponding epidemics in Australia[4] and Great Britain.[5, 6] In these countries, too, there appeared to be consecutive outbreaks although there was somewhat more overlapping. Strains of both types of virus were isolated in Australia. Among the Australian cases there were two and possibly three instances of consecutive infections with influenza B and A in the same individuals, and two such cases are included among those reported here. Evidence was also obtained from complement fixation tests done on serum pools that influenza A occurred sometime after the influenza outbreak of December, 1945, in New York State.[13] The isolation of influenza A virus in the Chicago area in December, 1945,[17] and serologic evidence that influenza A alone occurred in an outbreak at a boys' school thirty miles from Boston between Jan. 9 and Feb. 6[18] have also been recorded.

Symptomless infection associated with a significant rise in influenza virus antibodies was observed during this study in only one instance, namely the influenza A infection that occurred in Patient 59 (Table III) following the attack of clinical influenza which had given rise to influenza B antibodies. Not enough observations were made to determine the incidence of such symptomless infections, but the findings in Group VI suggest that they were not infrequent with the B virus and those in Groups IIB and VI suggest that they may also have occurred with some type of A virus.

As for the remaining serologically proved cases of influenza B (Table I) and A (Table IV), the rise in antibodies followed a characteristic attack of clinical influenza in every instance except in one patient of each of these groups. A similar observation was made in the Australian cases.[4] The symptomatology varied in the cases that were not associated with an antibody rise, both in those which occurred during the prevalence of influenza B and in those which occurred later when cases of influenza A were encountered. Some of those patients had symptoms of clinical influenza indistinguishable from those experienced by the patients showing definite antibody rises, but a large proportion of them had symptoms that were more like those of the common cold or were predominantly gastrointestinal. Furthermore, in the patients who had two distinct infections, one resembling clinical influenza and the other more like the common cold, the antibody response seemed to be temporally related to the former.

The failure of certain patients with typical symptoms of influenza to show antibody rises to the epidemic strain even when they have low titers during the acute phase has been noted frequently by others. This has been observed even in patients with typical illness from whom a virus has been isolated[5, 14] or who had been infected experimentally.[19]

The elevated titers of agglutinin inhibition of PR8 not accompanied by correspondingly elevated complement fixation titers are of some interest. They occurred in sera obtained after the middle of January from patients with clinical influenza who failed to show any significant antibody rise with the viruses used (Group IIB) and were also noted in the sera obtained from individuals picked at random late in February (Group VI). These may represent, in part, a response to infection with an influenza virus related to but not identical with PR8. Such a possibility is not inconsistent with the occurrence of typical rises of PR8 antibodies demonstrable by both agglutinin inhibition and complement fixation in other individuals, as noted in Table III.

The findings in Group VI suggest, in addition, that an appreciable proportion of the individuals in that group had had recent infection with influenza B, as evidenced by the elevated titers to those viruses demonstrable by both tests. The correlation of the elevated titers with the occurrence, time, and character of the infection as noted in the histories of these individuals was not very good. Some evidence was previously adduced to suggest that the WC and MF viruses were antigenically different from the Lee strain.[12, 15] Although similar antibody responses to all three strains were observed in most patients, there were sera which showed definite discrepancies both in the present cases and in those reported from Needham.[12] Further observations[15] have since been made to support the view that WC and MF are more closely related antigenically to the Australian strain BON,[20] and similar strains have been identified both in Australia[21] and Great Britain[5] during the 1945 epidemics in those countries.

SUMMARY AND CONCLUSIONS

Both influenza B and influenza A were prevalent in Boston during the winter of 1945-1946. Influenza B was responsible for the major outbreak that occurred in December, 1945, and no acute cases proved to be due to this virus were encountered after the first week in January. Cases of serologically proved influenza A were encountered almost entirely after the middle of January. Two cases were observed in which there was definite serologic evidence of consecutive infections first with influenza B and a few weeks later with influenza A. There was also some evidence of symptomless infections with viruses of both types.

Almost all of the patients in whose sera a rise in specific antibodies to one or another of the influenza viruses was demonstrated had respiratory and systemic symptoms which were characteristic of clinical influenza. Similar symptoms were noted during the same period in other individuals whose sera failed to show such a rise. A large proportion of the patients who failed to show a rise in influenzal antibodies, however, had an illness which was more like the common cold or had predominantly gastrointestinal symptoms.

REFERENCES

1. Anonymous: The Prevalence of Influenza B, Bull. U. S. Army M. Dept. 4: 369-370, 1945.
2. Anonymous: Prevalence of Communicable Diseases in the United States, Pub. Health Rep. **61**: 109-113, 1946.
3. Sigel, M. M., Hart, M. M., Hobbs, G., and Guthner, B.: Demonstration of Influenza Virus, Type B, in a Recent Outbreak of Upper Respiratory Infection, Science **102**: 646, 1945.

4. Burnet, F. M., Stone, J. D., and Anderson, S. G.: An Epidemic of Influenza B in Australia, Lancet **1**: 807-811, 1946.
5. Dudgeon, J. A., Stuart-Harris, C. H., Glover, R. E., and others: Influenza B in 1945-1946, Lancet **2**: 627-631, 1946.
6. Jackson, W. P. U.: Influenza B Among West Indians: Outbreaks in Bahamas and in England, Lancet **2**: 631-635, 1946.
7. Schwartz, S., Milstone, J. H., Bayless, M., and DeCoursey, E.: An Influenza Index on Periodic Determination of Average Antibody Titers of Population Samples, J. Immunol. **54**: 225-232, 1946.
8. Milstone, J. H., Lindberg, R. B., Bayliss, M., DeCoursey, E., and Berk, M. E.: 1945 Influenza B Epidemic in the Pacific Area, Mil. Surgeon **99**: 777-784, 1946.
9. Dalldorf, G., and Rice, C. E.: Influenza. A Preliminary State-Wide Survey Using Routine Blood Specimens, Am. J. Med. 2: 35-39, 1947.
10. Hirst, G. K., Vilches, A., Rogers, O., and Robbins, C. L.: Effect of Vaccination on the Incidence of Influenza B, Am. J. Hyg. **45**: 96-101, 1947.
11. Bruce, R. A., and Slavin, H. B.: A Study of an Outbreak of Influenza B in Rochester, New York, Am. J. M. Sc. **213**: 129-134, 1947.
12. Finland, M., Barnes, M. W., Meads, M., Ory, E. M., and Rubinstein, A. D.: Influenza B in Needham, Massachusetts, December, 1945. Serologic Studies in Cases and in Family Contacts, J. Lab. & Clin. Med. **32**: 367-378, 1947.
13. Rice, C. E.: Studies of the Complement Fixation Reaction in Virus Systems. II. Activities of Influenza Virus Antigens and Antibodies, J. Immunol. **56**: 343-356, 1947.
14. Finland, M., Barnes, M. W., and Samper, B. A.: Influenza Virus Isolations and Serological Studies Made in Boston During the Winter of 1943-1944, J. Clin. Investigation **24**: 192-208, 1945.
15. Barnes, M. W., Morgan, H. R., and Finland, M.: Isolation and Identification of Influenza Viruses During the Epidemic of December, 1945. To be published.
16. Finland, M., Ory, E. M., Meads, M., and Barnes, M. W.: Influenza and Pneumonia. Serologic Studies During and After an Outbreak of Influenza B, J. Lab. & Clin. Med. **33**: 32, 1948.
17. Forster, G. F., and Shaughnessy, H. J.: Isolation of Type A Influenza Virus in Recent Epidemic in the Chicago Area, Science **104**: 208-209, 1946.
18. Ingalls, T. H., Gallagher, J. R., and Enders, J. F.: An Outbreak of Influenza A in a Boys' School, New England J. Med. **235**: 786-788, 1946.
19. Henle, W., Henle, G., Stokes, J., Jr., and Maris, E. P.: Experimental Exposure of Human Subjects to Viruses of Influenza, J. Immunol. **52**: 145-165, 1946.
20. Beveridge, W. I. B., Burnet, F. M., and Williams, S. E.: The Isolation of Influenza A and B Viruses by Chick-Embryo Inoculation, Australian J. Exper. Biol. & M. Sc. **22**: 1-7, 1944.
21. Burnet, F. M., and Stone, J. D.: Serological Response to Influenza B Infection in Human Beings. Differentiation of Specific and Nonspecific Type Reactions, Australian J. Exper. Biol. & M. Sc. **24**: 207-211, 1946.

INFLUENZA AND PNEUMONIA

Serologic Studies During and After an Outbreak of Influenza B

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IT IS generally assumed that deaths from influenza are usually associated with pneumonia, as seemed evident from clinical and pathological findings during the pandemic of 1918. Excess mortality rates during periods of epidemic prevalence of influenza, however, are credited as much to increases in deaths from other causes as they are to increases in deaths from influenza and pneumonia.[1] Evidence for a direct relation of influenza virus infection to the occurrence or severity of pneumonia in individual patients is rather scant.

This relationship of influenza virus infection to bacterial and other pneumonias has been a matter of interest in this laboratory for the past few years. Some serologic evidence for an association of influenza A infection with cases of pneumococcal and, particularly, of staphylococcal pneumonia was obtained during the 1940-1941 outbreak.[2-4] Presumptive evidence for the presence of influenza A in the lung of a patient with a fulminating case of staphylococcal pneumonia was obtained at that time.[2, 4] Significantly elevated titers of influenza A antibodies also were demonstrated in cases of pneumonia of varied etiology which followed attacks of clinical influenza during the epidemic of 1943-1944.[5] In addition, influenza A virus was isolated from the lungs of five patients with fatal cases.[6] Two of the strains were from patients with the fulminating acute hemorrhagic and edematous type of staphylococcal pneumonia; a third was from a patient with lobar pneumonia in whom type 1 pneumococcus was obtained from the sputum, blood, and lungs; and cultures from the two remaining patients yielded no significant pathogenic bacteria and only a few alpha hemolytic streptococci from sputum and lungs. The onset of pneumonia occurred from one to three days after the onset of symptoms of clinical influenza in these five patients.

Other workers also have reported the isolation of influenza A virus from the lungs in acutely fatal cases of staphylococcal pneumonia; three such cases were reported from the 1936-1937 epidemic in England,[7] and one occurred in Philadelphia in January, 1939.[8] Various other types of lung changes were observed in patients with clinical influenza in the 1936-37 epidemic,[7, 9] but the commonest lung lesion in proved cases of influenza A infection in that outbreak was considered to be a "bronchiolitis."[9]

The occurrence of pneumonia during epidemics of influenza B also has been reported, but in these outbreaks the evidence for the association of the virus with the individual cases of pneumonia is scant. Four fatal cases of pneu-

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mococcal lobar pneumonia and four other patients with pulmonary involvement were observed during an epidemic of influenza B in a mental institution in Minnesota.[10] The presence of influenza B infection during an epidemic of type 1 pneumococcal pneumonia in Northville, N. Y., was demonstrated serologically.[11] In neither of these two outbreaks, however, was the relation of the influenza B virus infection established in any of the instances of pneumonia either serologically or by demonstration of the virus. Only one case has been reported prior to the epidemic of 1945 in which influenza B virus was isolated from the lung of a patient with a fatal case.[12] In this case, as in most of those previously mentioned in which influenza A virus was yielded from the lungs, there was a fulminating acute hemorrhagic and edematous type of *Staphylococcus aureus* pneumonia.

During a localized outbreak of influenza B which occurred in the Bahamas in 1945,[13] lobar pneumonia was recognized as a complication occurring from 0 to 14 days after the onset of clinical influenza in about 10 per cent of the cases. The relation of the virus was not established in any of the cases of pneumonia in this outbreak. Influenza B virus was isolated in Melbourne during the 1945 outbreak[14] from bronchial secretion obtained at autopsy from a patient with an acutely fatal case of staphylococcal pneumonia which began five days after the onset of influenza. This virus was also isolated in January, 1945, from throat gargling obtained "from a nurse in Surrey with a severe form of influenzal pneumonia."[15]

The epidemic of influenza that occurred in Boston in December, 1945, offered an opportunity to make some serologic studies of influenza antibodies in cases of pneumonia that occurred during and shortly after the time when influenza B, and later influenza A, infections were demonstrated in cases of clinical influenza.[16] The findings in these cases are reported here.

MATERIALS AND METHODS

Choice of Patients.—All of the patients chosen for this study were admitted to the Medical Wards of the Boston City Hospital, mostly during December, 1945, and January, 1946. Detailed histories were obtained with respect to the pneumonia and to any antecedent acute respiratory illness in an attempt to determine the time of onset of these infections as nearly as possible.

Bacteriologic studies of blood and sputum were carried out during the acute illness in almost all of the cases. Blood for serologic tests was obtained during the acute febrile stage of the illness and again at intervals thereafter. Patients from whom samples of serum were available within the first week of the onset of respiratory illness but not later are excluded here. The findings in the sera of several such patients, including five with fatal cases, are given elsewhere.[16] No attempts were made to isolate viruses from these patients and autopsies were not obtained in any of the fatal cases.

Clinical Findings.—The onset of pneumonia during or after a simple upper respiratory tract infection was usually marked by one or more of a number of manifestations: (1) the sudden occurrence of a brisk chill or of pleuritic pain

or both; (2) a recurrence of chills or chilly sensation; (3) the appearance of bloody or rusty sputum; (3) dyspnea; and (4) a change in the character and severity of the cough. The criteria for the classification of the upper respiratory tract infections were the same as those used in collateral studies of this outbreak reported elsewhere.[16, 17] The pneumonias were classified as either lobar or atypical (broncho-) pneumonia according to the clinical and x-ray findings. Some of the latter resembled characteristic cases of primary atypical ("viral") pneumonia.

Serologic Tests.—The viruses used were the PR8 strain of influenza A and the Lee strain of influenza B* and two strains WC and MF of influenza B isolated from uncomplicated cases of clinical influenza early in the course of this outbreak. The inhibition of chicken cell agglutination and the complement fixation tests were both carried out in every instance. The details of the methods used and the manner of recording the titers are the same as those used in other parallel studies.[17]

Tests for cold agglutinins were also carried out in all of these sera by the method usually employed in this laboratory.[18] The lowest final dilution of serum used in these tests was 1:10.

RESULTS

A total of sixty-nine patients was available for study. They have been divided for convenience into three groups: those in whom a significant rise in influenza B antibodies was demonstrated; those whose initial serum showed significantly elevated titers of influenza B antibodies; and those in whom the serologic findings failed to reveal any evidence of recent influenza virus infection. The findings in each of these groups will be considered separately.

Group I. Patients Whose Sera Showed a Significant Rise in Titer of Influenza B Antibodies.—There were eleven such patients; seven were women and four men and they ranged in age from 13 to 69 years. The relevant findings pertaining to the influenza and the pneumonia and the results of the serologic tests in these patients (1 to 11) are shown in the upper part of Table I. There was an illness which, from the history was considered to be clinical influenza in all but one of these cases. The first symptom of this illness began on November 30 in one patient and between December 18 and January 11 in the others. The onset of the pneumonia occurred on the same day in three cases, and one to ten days later in the others. In Patients 6 and 11 there were symptoms suggesting, respectively, an exacerbation of the original attack after five days and a second attack after six weeks.

The findings in the lungs were those of typical lobar pneumonia in two of these patients and cultures of the sputum yielded predominantly pneumococci in both. In all the others there was patchy consolidation. In Patient 1, the findings in the lungs closely resembled those of primary atypical pneumonia; sputum cultures yielded only alpha hemolytic streptococci. *Staph. aureus* was the predominant organism in repeated cultures in four other patients and pneumococci predominated in the remaining four, with staphylococci being recovered in moderate

*Both originally obtained from Dr. Thomas Francis, Jr., Ann Arbor, Mich.

numbers, particularly during convalescence from one of the latter patients. It is of interest that in Patient 1, who yielded no significant pathogens, and in the four patients in whom *Staph. aureus* predominated, pneumonia began either on the same day (three cases) or within two days of the onset of influenza.

There was slight to moderate leucocytosis with polynuclear predominance during the febrile stage of the pneumonia in eight of these patients. In the other three the total counts averaged between 4,000 and 9,000, the lowest counts being obtained in one of the patients with staphylococcal pneumonia. Blood cultures were negative in all of the eleven patients and cold agglutinins could not be demonstrated in any of the sera. All but two of the patients were treated with sulfadiazine or penicillin or both, and the response to this treatment varied from fair to excellent. All recovered without complications.

The serologic findings in these cases were similar in every respect to those found in uncomplicated cases of influenza B which were studied at the same time.[16, 17] The initial titers of influenza B antibodies were low in every subject except Patient 5, and in that patient the initial serum was obtained eight days after the onset of influenza. In every case, including Patient 5, there was at least a fourfold and usually a much greater rise in titer of influenza B antibodies demonstrable by both the agglutinin inhibition and complement fixation tests. Similar results were obtained with the Lee strain and with the two recently isolated strains in the sera of four patients who were tested with all three strains.

None of these patients showed a significant rise in titer of antibodies to PR8. Agglutinin inhibition titers of 64 or higher were found in the sera of four of the patients and complement fixation titers of 32 or higher were obtained in the sera from these four patients only.

The time relationships between the influenza B antibody response and the onset of the infections in three patients are worthy of comment. In Patient 9 the initial titers of agglutinin inhibition of the influenza B viruses were all low; this serum was obtained thirteen days after what was considered to be the onset of clinical influenza. A significant rise in these antibodies was demonstrated in serum obtained five days later, or eight days after the onset of the pneumonia. Since rises in titers have usually been found in adults by the eighth day, these findings suggest the possibility that the initial illness either was not influenza B or else it failed to elicit an antibody rise until after pneumonia began. The findings in Patient 10 were similar, but in this case there were significantly elevated titers of PR8 antibodies in the initial serum which was obtained ten days after the onset of influenza and five days after what was considered to be the onset of pneumonia. The rise in B antibodies was demonstrated in serum obtained five days later. In Patient 11 there were two illnesses six weeks apart and from the history both were considered to be clinical influenza. The pneumonia began on the same day as the second attack and the rise in influenza B antibodies occurred between three and eleven days later.

Group II. Patients With Serologic Evidence of Recent Infection With Influenza B.—There were twenty-five patients in this group (12 to 36 in Table I); thirteen were men and twelve were women and they ranged in age from 13 to 80 years. A history considered to be that of clinical influenza was obtained in

TABLE I. RELEVANT DATA IN CASES OF PNEUMONIA WITH SEROLOGIC EVIDENCE OF INFLUENZA B INFECTION

PATIENT	SEX	AGE	CLINICAL INFLUENZA		SALIENT FEATURES OF PNEUMONIA								AVERAGE TITER OF INFLUENZA ANTIBODIES								
									BACTERIOLOGY					PR8		LEE		WC		MF	
			DATE OF ONSET	SEVERITY	DATE OF ONSET	TYPE OF LESION	LOBES INVOLVED	WHITE BLOOD COUNT	BLOOD	SPUTUM*	ANTIBACTERIAL THERAPY	RESPONSE	DATE OF SERUM	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION
1	M	60	11/30	++	11/30	B	Rl	8.	0	S. vir.?	0		11/30	160	34	20	3				
													12/14	112	20	640	1792				
2	F	19	12/18	++	12/20	B	RlLl	19.-9.	0	S. au.	P + S	+	12/24	12	6	12	4				
													1/ 3	16	5	512	28				
3	M	69	12/21	+++	12/21	B	Bilat.	12.-23.	0	S. au.	P + S	+	12/27	12	12	48	16				
													1/ 3	16	8	512	640				
4	M	57	12/23	+++	12/23	B	Rl	6.-4.	0	S. au.	P + S	++	12/26	24	5	3	2				
													1/ 3	16	5	320	320				
5	F	14	12/24	+++	12/25	B	RuLl	10.-2.5	0	S. au.	P + S	+++	1/ 1	64	40	64	48				
													1/14	96	28	1536	192				
6	F	43	12/24	+	12/29	B	Rm	17.-11.	0	Pn. 3	S	++	1/ 2	8	2	20	41	12	48	24	24
			12/29	+++									1/ 8	8	3	56	202	48	192	64	224
7	F	13	12/24	+	12/29	B	Rl	20.	0	Pn. 19	P + S	+	12/29	19	2	3	2				
										S. au.			1/ 3	11	2	768	320				
													1/16	16	2	1280	160				
8	F	24		0	12/29	B	Rl	15.-7.	0	Pn. 3	S	?	1/ 1	32	48	3	2	2	2	2	2
										S. vir.?			1/14	64	56	14	18	8	24	16	46
9	F	15	12/27	+++	1/ 6	L	Ru	22.-12.	0	Pn. 15	P + S	++	1/ 9	16	Ac	10	Ac	2	Ac	4	Ac
													1/14	16	Ac	39	Ac	16	Ac	32	Ac
10	M	60	1/ 3	++	1/ 8	L	Ru	9.-6.	0	Pn. 7	S	+++	1/13	192	96	6	2				
													1/18	128	64	192	80				
11	F	25	12/ 1	++	1/11	B	Rl	10.-14.	0	Pn. 19	0		1/14	27	18	5	19	2	24	2	24
			1/11	++									1/22	28	24	80	36	16	48	24	48
12	F	60	12/ 6	++	12/ 6	B	Ru	40.-18.	0	S. au.	P + S	+	12/26	32	20	192	96				
													1/ 8	32	20	512	80				
13	M	47	12/20	+	12/21	L	Ru	15.-10.	Pn. 8	S. au.	P	++	12/27	16	12	272	192	32	256	192	320
										(late)			1/ 3	16	10	268	256	48	320	192	640
14	F	67		0	12/22	L	Ll	7.-12.	S. alb.?	S. vir.?	S	+++	12/28	6	2	2048	192				
													1/ 4	8	2	2048	192				
15	F	30	12/16	+++	12/23	B	Rml	9.-11.	Cont.	--	P + S	++	12/28	32	6	256	640				
													2/21	32	6	64	112				
16	M	21	12/17	++	12/23	L	La	13.	0	Pn. 3	S	+++	12/28	32	40	2048	320				
17	M	17	12/17	++	12/25	B	Rl	10.-3.	0	S. hem.	P	+++	12/25	24	48	512	808	512	768	1024	896
													1/ 2	24	20	512	752	512	768	1024	768

18	F	35	12/20	++	12/26	B	RlLl	17.	0	S. hem.	P + S	++	1/ 2	17	2	512	1280			
													2/13	12	3	192	440			
19	M	36	12/23	++	12/27	L	Rum	6.	0	Pn. 7	P	+++	12/31	4	3	384	640			
													1/ 9	4	3	384	384			
20	M	62	12/25	+	12/28	B	RlLl	16.-13.	0	Pn. 3	S	+++	1/ 5	6	24	18	32	16	40	40	64
													1/10	24	20	56	48	32	56	64	80
21	F	52	12/ 3	++	12/28	L	Lu	16.-10.	Pn. 4	Pn. 4, S. au.	P + S	+++	12/30	6	6	88	272	128	384	160	256
			12/25	++									1/ 9	8	5	88	240	64	224	160	192
22	M	35	12/27	++	12/28	L	Ll	13.	Pn. 7	Pn. 7	P	+++	2/13	16	5	60	116	64	128	80	112
													1/10	32	10	128	80				
23	M	31	?	C	12/29	B	Rm	8.	0	S. vir.?	0		1/14	16	10	512	112				
													1/ 5	24	12	288	40	32	40	64	64
24	F	36		0	12/30	L	Rl	8.	0	Pn. 8	S	++	1/ 9	24	14	96	40	24	28	96	56
													1/ 2	24	20	192	80				
25	F	21	1/ 1	++	1/ 1	B	RlLu	18.	S. au.	--	P + S	++	1/ 9	24	10	384	96				
													1/ 8	3	2	192	192				
26	M	50		0	1/ 1	L	Llu	12.-14.	0	Pn. 7, Pn. 17	S	+++	1/14	4	2	512	320				
													1/ 7	4	3	48	112				
27	M	56	1/ 1	++	1/ 4	L	Rl	13.	Pn. 5	Pn. 5	P + S	+++	1/14	3	5	48	192				
													1/ 8	16	3	288	54	128	40	160	40
28	F	21	12/27	++	1/ 5	L	Rml	21.-24.	0	--	S	+++	1/14	8	2	336	60	160	64	256	96
													1/ 8	6	3	96	448				
29	F	50	12/25	++	1 /6	B	Rl	21.	0	--	P + S	+++	1/14	4	3	96	640				
													1/ 8	2	2	20	272	512	640	1024	1280
30	F	54	12/27	+++	1/ 7	B	RlLl	12.	0	Pn. 22, Pn. 10	S	++	1/14	2	2	88	208	512	384	1024	1280
													1/ 9	2	2	128	80				
31	M	35	12/29	+	1/ 9	L	Rl	18.	0	Pn. 1	S	++	1/15	2	2	96	96				
													1/12	64	16	2048	128				
32	M	13	1/ 4	++	1/12	B	Rl	14.-19.	0	S. vir.?	P	++	1/18	283	13	2048	160				
													1/12	64	56	1536	640	1024	512	512	384
33	M	63	1/12	++	1/12	B	Lul	10.-4.	0	S. au., Pn. 12	P + S	++	1/18	64	56	2048	640	768	512	512	384
													1/18	4	12	768	112				
34	F	36	1/11	+	1/19	L	Rml	16.	0	Pn. 1	P + S	+++	1/26	4	12	1024	160				
													1/22	3	10	512	768				
35	F	65	1/ 5	C	1/19	L	Rml	22.-13.	0	Pn. 3	P + S	++	1/29	6	6	768	640				
													1/19	4	10	144	21	32	28	96	28
36	M	80		?	?	B	Bilat.	13.	0	--	S	+++	1/28	8	3	64	17	32	24	64	24
													1/ 8	3	5	160	17	96	28	128	24
													1/16	2	5	168	22	96	40	128	40

Severity: +, ++, +++, increasing severity of symptoms characteristic of influenza; C, coryza and/or cough predominating without fever or systemic symptoms; 0, no symptoms; ?, history uncertain. Type of lesion: L, lobar distribution of consolidation; B, patchy (atypical) infiltration. Lobes involved: R, right; L, left; u, upper; m, middle; l, lower; Bilat., patchy infiltration in both lungs. White blood count: range (in thousands) during febrile course of the pneumonia. Bacteriology: 0, no growth; ---, not done or not available; Pn., pneumococcus (the numeral represents the type); S. au., *Staphylococcus aureus;* S. alb., *Staphylococcus albus;* S. hem., *Streptococcus hemolyticus* (beta); S. vir., *Streptococcus viridans* (alpha hemolytic); Cont., contaminant; ?, doubtful significance. Antibacterial therapy: 0, none; P, penicillin; S, sulfonamide (sulfadiazine in most instances). Response: +++, excellent (crisis within twenty-four hours); ++, good (afebrile in twenty-four to seventy-two hours with corresponding improvement); +, probably beneficial or slow improvement. Complement fixation: Ac, anticomplementary.

*Predominant organism.

Excluded from this table are three patients with pneumonia and serologic evidence of recent infection with influenza A (and not with influenza B). They are included as Numbers 57, 63, and 64 in Table III of a previous communication.[16]

nineteen of these patients, including one who had two similar attacks three weeks apart; two others had an illness which was more like a common cold, while no history of an antecedent illness distinguishable from pneumonia could be elicited in the four remaining patients. The onset of these illnesses occurred between December 3 and January 12, and in all but three instances they began between December 15 and January 5.

The interval between the onset of influenza or cold and pneumonia was seven days or less in twelve cases and from eight to fourteen days in eight others. The pulmonary lesion in thirteen patients was that of typical lobar pneumonia involving a single lobe or two contiguous lobes. Pneumococci predominated in cultures of the sputum in eleven of these patients and were also grown from the blood of four of them; in one of the latter the sputum cultures yielded predominantly *Staph. aureus* during convalescence. Cultures of sputum in one instance yielded only alpha hemolytic streptococci, and none were made in one case.

The remaining twelve patients had atypical pulmonary lesions which involved chiefly a single lobe in five, two contiguous lobes in two, and both lungs (mostly the lower portions) in the other five. *Staph. aureus* was obtained from a blood culture of one of these patients and was predominant in the sputum cultures of two others, including one which yielded pneumococci as well; beta hemolytic streptococci were the predominant organisms in the sputum in two patients and pneumococci predominated in two others. Cultures in the remaining five cases were either inadequate or yielded no significant pathogens.

The leucocyte counts were elevated during the febrile course in most of the patients with atypical pulmonary lesions and were similar in those with lobar and atypical pneumonia. Total counts of 8,000 or lower were obtained some time during the febrile course in two of the former and in three of the latter. In the others the total counts ranged between 12,000 and 40,000, with polynuclears predominating in every instance. None of the patients in this group showed elevated titers or rises in titers of cold agglutinins.

All but one of the patients in this group were treated with sulfadiazine, penicillin, or both and responded favorably to this treatment. They all recovered and became essentially afebrile within twenty-four to seventy-two hours after the treatment was started.

The initial serums were obtained in almost all of these cases between the sixth and fourteen day after the onset of the initial illness and within the first week after the onset of the pneumonia. The titers of influenza B antibodies in these initial sera were significantly elevated and about equally so in both tests and for each of the B strains used. There were six patients in whom there were suggestive or definite rises in these titers on repeated tests with one or more of the B strains. The initial serum in four of these six patients was obtained on or before the seventh day after the onset of clinical influenza. In three other patients the later sera showed a definite drop from high titers; the initial serum was obtained on the twelfth day in one and on the thirteenth day in the second, while the third patient had two distinct illnesses which began twenty-seven and five days, respectively, before the first blood was taken. In the latter case a suggestive drop was noted in the titer of the second blood obtained ten days after the first one.

No significantly elevated titers or rises in titers of antibodies to the PR8 strain were obtained in this group except in Patient 31. In this patient a slightly greater than fourfold rise was elicited by the agglutinin inhibition tests, while the complement fixation tests with the same virus yield low titers and no rise.

Group III. Patients Lacking Serologic Evidence of Influenza Virus Infection.—The relevant findings in thirty-three such patients are listed in Table II. The clinical features of this group resembled those of the two previous groups in some respects but showed some important points of difference. The age distribution, for example, was similar but the sex distribution in the present cases differed from that of the other groups and was more like that usually found in acute bacterial pneumonias in adults. The patients ranged in ages from 14 to 75 years and there were twice as many men as women.

A larger proportion of the antecedent illnesses in this group were simple colds. Thus, of twenty-nine patients from whom a history of antecedent illness distinct from the pneumonia could be elicited, that illness was classified as clinical influenza in twelve, as a cold in twelve, and as a cold followed five to twenty-six days later by clinical influenza in five. The interval between the onset of the illness that was characterized as clinical influenza and the onset of pneumonia was two days or less in twelve cases and five to ten days in five cases. The interval between the onset of the cold and that of the pneumonia was less than two days in only two patients, but five to ten days in six, and eleven to twenty-eight days in eight.

The pulmonary lesion was classified as typical lobar pneumonia in nineteen patients, involved a single lobe in fourteen, and two contiguous lobes in the other five. The predominant organism in the sputum was *Staph. aureus* in one of these patients and the same organism was obtained from the blood culture of that patient; pneumococci predominated in the sputum of seventeen, including one with a positive blood culture; and the bacteriology was inadequate in one case.

The remaining fourteen patients had atypical pulmonary lesions involving mostly the lower portions of the lung—one side in eight and bilaterally in six. The predominant organisms varied more than among the lobar pneumonias: *Staph. aureus*, beta hemolytic streptococci, pneumococci, and Friedländer's bacilli each were found in two or three cases, alone or in combination with one of the others. A positive blood culture for hemolytic streptococci was obtained in one case. In two patients no adequate bacteriological studies were made and in three others the sputum cultures yielded predominantly alpha hemolytic streptococci but no significant pathogens.

Cold agglutinins were demonstrated (a rise from < 10 to 80) in one of the latter but in none of the other thirty-two patients in this group. The leucocyte counts during the febrile period in this group were similar to those found in the other groups. Total counts of 5,000 or less were obtained in three cases, between 6,000 and 10,000 in six, and from 11,000 to 28,000 in the others. Sulfadiazine, penicillin, or both were used in the treatment of all but three of these patients, with good effects in most instances. There were two deaths in this group, one a 75-year-old patient with pneumococcal pneumonia complicated by congestive heart failure (Patient 61) and the other a 65-year-old patient with Friedländer's pneumonia.

TABLE II. FINDINGS IN CASES OF PNEUMONIA LACKING SEROLOGIC EVIDENCE OF INFLUENZA B INFECTION

PATIENT	SEX	AGE	CLINICAL INFLUENZA		SALIENT FEATURES OF PNEUMONIA								AVERAGE TITER OF INFLUENZA ANTIBODIES									
									BACTERIOLOGY					PRS		LEE		WC		MF	
			DATE OF ONSET	SEVERITY	DATE OF ONSET	TYPE OF LESION	LOBES INVOLVED	WHITE BLOOD COUNT	BLOOD	SPUTUM*	ANTIBACTERIAL THERAPY	RESPONSE	DATE OF SERUM	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION
37	M	51	12/ 4	C	12/ 9	B	Rl	9.-18.	0	S. au., H. inf.	S	+	12/20	8	3	6	3				
			12/ 9	++									1/ 2	8	3	6	2				
38	M	41		0	12/10	L	Rum	12.	0	Pn. 29, H. inf.	P	+	12/17	3	5	2	3	2	2	2	2
													1/ 2	6	Ac	2	Ac	2	Ac	2	Ac
39	F	41	12/ 3	C	12/12	L	Rl	26.	0	Pn. 15, S. au.	P	+++	12/19	3	10	8	6				
													12/26	8	7	8	7				
40	M	38	?	C	12/12	L	Rl	6.	0	Pn. 4	P	+++	12/24	12	4	2	2				
													1/ 3	12	4	3	2				
41	M	15	12/ 4	C	12/15	L	Ll	22.	0	Pn. 2	P + S	+++	12/20	8	5	2	2				
			12/14	++									12/26	6	5	2	2				
42	F	14	12/19	C	12/20	B	Bilat.	8.	0	S. vir., S. au.	P + S	+	12/26†	24	10	24	8	5	14	20	12
													1/ 2	24	8	24	11	5	12	20	12
43	M	54	12/20	++	12/20	B	Bilat.	21.-5.	0	--	S	0	12/28	16	3	8	2				
													1/ 4	24	2	6	2				
44	M	60	12/ 2	C	12/21	L	Rl	8.-6.	0	Pn. 8	P	++	12/26	6	12	32	14	16	20	32	14
													1/ 4	6	6	32	9	16	16	48	12
45	M	48	12/10	++	12/19	L	Rml	28.-13.	0	Pn. 8	P	++	12/26	4	4	4	24				
													1/ 5	4	3	4	20				
46	M	34	12/20	+++	12/26	L	Rum	20.-15.	S. au.	S. au.	P + S	++	12/26	24	3	7	6	2	6	12	10
													1/ 3	32	3	7	5	2	3	12	8
47	M	54	12/25	++	12/26	L	Ll	18.	0	Pn. 12	P + S	++	1/ 2	16	6	3	5	2	7	2	10
													1/ 9	16	3	7	5	2	10	4	12
48	F	35	12/ 1	C	12/28	L	Rml	13.	0	Pn. 8	S	+++	12/28	24	44	40	48			32	56
			12/27	++									1/ 5	32	55	80	56			32	56
49	F	47	12/13	C	12/27	B	Rl	13.	--	--	0		12/28	8	4	3	2				
													1/ 3	8	4	3	2				
50	M	37	12/22	C	12/27	B	Rl	27.	S. hem.	S. hem.	P	+	1/ 3	455	8	8	3				
							Emp.						1/ 8	455	4	16	2				
51	M	60		0	12/29	B	Ll	6.	0	Pn. 21	S	++	1/ 3	4	2	3	2				
													1/10	6	2	3	2				

52	M	28	12/28	C	12/29	L	Rl.	14.-18.	0	Pn. 1	P	+	1/ 5	12	6	12	3					
53	M	24	12/20	++	12/30	L	Emp. Lu	9.-6.	0	--	S	++	1/12	12	6	12	3					
54	M	46	12/31	+++	12/31	L	Rl	16.	0	Pn. 2	S	++	1/ 3	10	6	6	12					
55	F	17	12/31	++	12/31	L	Ll	14.	0	Pn. 8	S	+++	1/ 9	16	7	3	12					
56	M	45		0	1/ 2	L	Rl	18.	0	Pn. 7	S	+++	1/ 5	40	11	24	7	6	6			
57	M	54	12/17	C	1/ 6	L	Ll	14.-25.	Pn. 1	Pn. 1	P + S	+	1/16	40	9	36	7	6	6			
58	F	38	12/28	C	1/ 8	L	Lul	27.	0	Pn. 2	P + S	+++	1/ 5	16	14	10	7	4	12	5	14	
59	F	55	1/ 7 1/ 9	++ +++	1/ 9	B	Bilat.	4.-5.	0	S. vir. ?	P	+++	1/11	16	12	10	5	4	6	8	6	
60	M	54	1/ 7	++	1/ 9	L	Ll	12.-8.	0	Pn. 7	S	++	1/12	64	2	24	2					
61	F	75	1/ 5	C	1/12	B	Rl	17.	0	Pn. 17	P	++	1/18	64	2	32	2					
62	F	65	1/ 9	++	1/14	B	Bilat.	7.-16.	0	K. pn. A	P	D	1/ 8	12	2	24	3					
63	F	61	1/ 2 1/14	C ++	1/14	B	RlLl	25.-9.	0	S. vir. ?	P	+	1/14	16	2	16	2					
64	M	72		0	1/16	B	Bilat.	2.6-1.3	0	Pn. 3, K. pn. A	P + S	+	1/10	32	24	40	5					
65	M	48	1/10	C	1/17	L	Ru	8.-1.7	0	Pn. 8	P + S	+++	1/16	64	14	16	2					
66	F	66	1/ 7	C	1/18	B	Rl	10.	0	S. vir. ?	P	0	1/17	8	5	4	5					
67	M	62	2/ 1	++	2/ 1	B	Rl	25.-10.	0	S. hem., Pn. 6	P	+	1/26	8	3	4	3					
68	M	48	1/27	++	2/ 3	B	Rl	11.-9.	0	S. au., S. hem.	P + S	+	1/14	48	44	15	11					
69	M	22	2/ 9	C	2/16	L	Rl	16.	0	Pn. 4	P + S	+++	1/22	112	38	15	15					
													1/19	4	2	12	2					
													1/26	4	2	6	2					
													1/17	16	2	6	2					
													1/26	12	2	12	2					
													1/16	8	3	12	3					
													1/23	6	5	8	3					
													1/22	12	6	48	6					
													1/28	12	5	32	3					
													1/22	4	10	12	2					
													1/28	6	10	3	2					
													1/22	8	3	16	2					
													2/ 4	4	5	8	3					
													2/ 8	24	5	48	2					
													2/14	16	2	32	2					
													2/ 7	56	3	8	2					
													2/14	100	4	12	2					
													2/21	24	2	76	6	8	14	16	14	
													2/28	24	2	76	8	8	12	16	14	

Severity: +, ++, +++, increasing severity of symptoms characteristic of influenza; C, coryza and/or cough predominating without fever or systemic symptoms; 0, no symptoms. Type of lesion: L, lobar distribution of consolidation; B, patchy (atypical) infiltration. Lobes involved: R, right; L, left; u, upper; m, middle; l, lower; Bilat., patchy infiltration in both lungs; Emp., empyema. White blood count: range (in thousands) during febrile course of the pneumonia. Bacteriology: 0, no growth; --, not done or not available; Pn., pneumococcus (the numeral represents the type); S. au., *Staphylococcus aureus*; S. alb., *Staphylococcus albus*; S. hem., *Streptococcus hemolyticus* (beta); S. vir., *Streptococcus viridans* (alpha hemolytic); K. pn. A., Friedländer's bacillus, type A; H. inf., *Hemophilus influenzae*; Cont., contaminant; ?, doubtful significance. Antibacterial therapy: 0, none; P, penicillin; S, sulfonamide (sulfadiazine in most instances). Response: +++, excellent (crisis within twenty-four hours); ++, good (afebrile in twenty-four to seventy-two hours with corresponding improvement); +, probably beneficial or slow improvement; 0, no apparent benefit; D, died. Complement fixation: Ac, anticomplementary.

*Predominant organism.

†Cold agglutinin titers: 11/26, <10; 12/2, 80.

The initial serum was obtained during the first week after the onset of pneumonia in twenty-eight patients and early in the second week in the other five. In almost every instance this was more than seven days after the onset of the antecedent respiratory illness and a second serum was obtained about a week or more later. Any significant elevation or rise in influenza antibody titers resulting from these infections should, therefore, have been manifested in the samples of serum that were tested.

None of these patients had significantly elevated titers or rises in titers by either test with any of the B viruses, with the possible exception of Patient 48; the titers in that patient were of borderline significance but no definite rise was elicited. Essentially the same was true of the PR8 antibodies. Titers of agglutinin inhibition greater than 64 were obtained in only three patients, but the corresponding complement fixation titers were elevated in only one. Interestingly enough, only one of these three cases occurred after the middle of January when influenza A infection was demonstrated in other uncomplicated cases.[16]

TABLE III. SUMMARY OF RESULTS OF SEROLOGIC TESTS WITH PR8 AND LEE VIRUSES IN THREE GROUPS OF CASES

GROUP*	VIRUS	TEST†	NUMBER OF CASES	TITER‡										AVERAGE
				4	8	16	32	64	128	256	512	1,024	2,048	
I Early	PR8	AI	11	0	1	3	4	1	0	2				51
Late	PR8	AI	11	0	1	5	1	1	3	0				47
II Early	PR8	AI	25	9	4	2	8	2	0	0	0			17
Late	PR8	AI	24	8	4	4	6	1	0	0	1			26
III Early	PR8	AI	33	6	7	9	6	4	0	0	1			31
Late	PR8	AI	33	3	10	9	5	3	2	0	1			36
I Early	PR8	CF	10	2	2	1	1	3	1					26
Late	PR8	CF	10	2	3	0	3	2	0					22
II Early	PR8	CF	25	9	3	7	3	3						13
Late	PR8	CF	24	9	5	6	3	1						10
III Early	PR8	CF	33	15	9	6	1	2						8
Late	PR8	CF	32	17	9	4	1	1						7
I Early	Lee	AI	11	3	2	2	2	2						18
Late	Lee	AI	11	0	0	1	0	2	1	1	3	1	2	471
II Early	Lee	AI	25	0	0	0	2	1	4	6	7	1	4	515
Late	Lee	AI	24	0	0	0	0	5	4	2	8	2	3	516
III Early	Lee	AI	33	8	8	7	5	4	1					17
Late	Lee	AI	33	9	8	8	5	1	2					16
I Early	Lee	CF	10	6	0	1	1	2						14
Late	Lee	CF	10	0	0	0	2	1	1	2	2	1	1	363
II Early	Lee	CF	25	0	0	0	3	2	7	3	4	5	1	301
Late	Lee	CF	24	0	0	0	2	3	5	7	3	4	0	246
III Early	Lee	CF	33	19	10	2	1	1						6
Late	Lee	CF	32	21	6	3	1	1						6

*Group designations are the same as those used in the text.

†AI, Inhibition of chicken cell agglutination; CF, Complement fixation.

‡Each category includes intermediate titers up to the next preceding one, for example, 4 = 4 or less; 8 = 5 to 8; 16 = 9 to 16, etc.

Summary of the Serologic Findings.—The distribution of titers of antibodies to the PR8 strain of influenza A and the Lee strain of influenza B obtained in the sera of each of these three groups of patients is shown in Table III. The results with the PR8 virus were essentially the same in all three groups. These

titers were in the low range except for a few slightly elevated titers demonstrated by the agglutinin inhibition test and fewer by the complement fixation test. The average titers with both tests were low and they would be appreciably lower were it not for the aberrant elevated titers. In each group the distribution of the titers and the average titers of PR8 antibodies were essentially the same in the early and in the late sera.

The distribution of the titers of antibodies to the Lee virus and the average titers of these antibodies were strikingly different in each of the three groups. In Group I the titers of the early sera were essentially in the low range, whereas the later sera were shifted markedly toward the high range. The average titer of the later sera was about twenty-six times that of the early sera. In these respects the findings were essentially the same in the agglutinin inhibition and in the complement fixation tests.

In Group II, the distribution of titers and the average titers in the early and late sera by both tests closely resembled those noted in the late sera in Group I. The results in both the early and late sera in Group III, on the other hand, paralleled those found in the early sera in Group I. The findings in Group I are very similar to those noted in the uncomplicated cases of influenza which occurred prior to mid-January and which were proved serologically to be infections with influenza B; those in Group III closely resembled the results noted in the cases of uncomplicated clinical influenza and in the simple acute respiratory infections that occurred at the same time and later, and which failed to elicit an antibody response to any of the influenza viruses used.[16] This failure to elicit an antibody response was not related to the finding of elevated titers in either instance. The findings in Group II may, therefore, be considered as strongly indicating recent infection with influenza B virus in those patients.

TABLE IV. AVERAGE TITER OF ANTIBODIES TO THREE STRAINS OF INFLUENZA B IN THREE GROUPS OF CASES

	AVERAGE TITERS								
	LEE			WC			MF		
GROUP*	NUMBER	AI†	CF†	NUMBER	AI†	CF†	NUMBER	AI†	CF†
I Early	11	18	14‡	4	5	25‡	4	8	17‡
Late	11	471	363	4	21	89‡	4	34	107‡
II Early	25	515	301	10	250	278	11	312	310
Late	24	516	246	10	225	232	11	312	323
III Early	33	17	6	8	6	10	8	15	17
Late	33	16	6‡	8	6	9‡	8	18	17‡

*The group designations are the same as those used in the text.
†AI, Inhibition of chicken cell agglutination; CF, complement fixation.
‡These averages are based on one serum fewer than indicated.

The number of patients whose sera were tested with the epidemic strains of influenza B was rather small. The results with these strains, however, paralleled those obtained with the Lee strain except that the titers in the late sera and the rises in these titers in Group I were somewhat lower with the epidemic strains. This discrepancy, however, is more apparent than real since the few sera chosen for the tests with these strains also had similarly low titers of Lee antibodies, as is evident from Table I. The average titers obtained with the three strains of influenza B in the three groups of patients are shown in Table IV.

COMMENT

The cases of pneumonia included in this study resembled the usual run of such cases observed in this hospital during the same season of each year. Possibly the number of cases of staphylococcal pneumonia was greater than is encountered during periods when influenza is not prevalent. Whether or not there was a real excess of pneumonia cases limited essentially to the period of epidemic prevalence of influenza could not be determined easily because of the great fluctuation in the number of cases observed from year to year.

The data presented indicate clearly that a large proportion of bacterial and other pneumonias that were encountered during and for a brief period after the time when influenza B was prevalent in this community gave evidence of infection with that virus. In this respect, these findings and the isolation of influenza B virus from sputum[15] and lungs[14] of individuals with severe cases of pneumonia in other areas confirm and extend, for an influenza B epidemic, the observations previously made during epidemics of influenza A.

The numbers of patients with pneumonia included here and those with uncomplicated influenza or other acute respiratory illness that were studied during the same period[16] were not very large. It is of interest to note, nevertheless, that roughly one-half of the patients from whom sera were obtained at the proper time gave serologic evidence of infection with influenza virus. Furthermore, this proportion was essentially the same whether or not the influenza or simple respiratory illness was followed by pneumonia.

During the present outbreak it was not possible to predict with certainty from the clinical features of the illnesses whether or not an influenza antibody response would be demonstrated. Except in rare instances, however, the illnesses in those patients in whom influenza antibody responses were elicited conformed to the clinical picture classically associated with clinical influenza. On the other hand, a significant proportion of the illnesses which occurred at the same time and in which evidence of virus infection could not be demonstrated was more like the illness usually classified as a common cold. Looked at from another point of view, a majority of the patients with characteristic symptoms of clinical influenza during this epidemic gave serologic evidence of infection with influenza B, whereas only an occasional patient who had symptoms of a common cold at this time gave serologic evidence of infection with that virus or with influenza A. This failure cannot be considered as proof that either of these types of illness was not due to influenza virus. That aspect was discussed in a previous paper.[16]

In all of the cases of pneumonia which yielded serologic evidence of infection with influenza B, the onset of the antecedent clinical influenza occurred within the period of epidemic prevalence of influenza B in this area. In the great majority of the cases this was between the second week in December and the first week in January.

No definite evidence of infection with influenza A was obtained among the cases of pneumonia included here. Three such cases were included in a previous paper.[16*] These three patients had symptoms of clinical influenza which began

*They are listed as Numbers 57, 63, and 64 in Table III of that paper.[16]

on January 25, February 1, and February 2, respectively. The onset of the pneumonia was on February 12 in the first, February 6 in the second, and February 2 in the third. No significant bacterial pathogen was obtained in the first case; clinically that case resembled primary atypical pneumonia. The other two were typical cases of pneumococcal lobar pneumonia due to type 5 and type 4 pneumococcus, respectively.

Another interesting feature of the present cases was the difficulty sometimes encountered in differentiating between the onset of the influenza and that of the pneumonia. Indeed, in some of the cases the onsets, at least from a clinical point of view, occurred almost simultaneously. In the others, varying intervals elapsed before the pneumonia began but these intervals were usually shorter after illnesses that were characteristic of clinical influenza than they were after illnesses that resembled the common cold. That seemed to be true irrespective of whether or not the clinical influenza was associated with an antibody response to the virus.

The significance of the findings of evidence of influenza virus infection with respect to the occurrence, incidence, or severity of bacterial and other pneumonias is by no means clear. The findings in some cases suggest that the pulmonary lesion is due primarily, if not entirely to the virus, but such cases are rare. In most of the cases, the character of the pulmonary lesion, the clinical course of the disease, and the response to antibacterial therapy is characteristic of infection with the predominant bacteria found in the sputum or lungs and does not seem to be markedly influenced by the antecedent influenza virus infection. Some cases, particularly those in which hemolytic *Staph. aureus* is the predominant or only bacterial invader, may be exceptions.

The situation may be different under other conditions, such as those which prevailed during the influenza pandemic of 1918. The nature of the primary etiologic agent of that pandemic is not known, but the incidence and severity of the complicating pneumonias at that time were different from those encountered during any of the epidemics which were known to be caused by varieties of influenza A or B. A close resemblance, however, was demonstrated between the staphylococcal pneumonias which occurred during the influenza A outbreak of 1940-1941 and the pulmonary complications that occurred in certain circumscribed areas during the pandemic.[3, 4] There are very little data available from which one may determine whether the nature of the virus or of the bacteria or the proper combinations of virus and bacteria were responsible for some of the varieties of pneumonia that were encountered during that great pandemic.

SUMMARY AND CONCLUSIONS

Some of the relevant clinical findings in sixty-nine cases of bacterial and other pneumonias that were studied during and shortly after the epidemic of influenza B which occurred in Boston in December, 1945, are presented. Serologic tests for antibodies to influenza A and B virus, including two epidemic strains of the latter, were carried out in sera obtained from these patients during and after the acute phase of the pneumonia.

About one-half of the cases that occurred during the period of epidemic prevalence of influenza B, irrespective of the clinical character of the pneumonia

or of the bacteriologic findings, yielded serologic evidence of infection with influenza B virus. Almost all of the patients from whom serologic evidence of influenza B infection was obtained had an illness which was characteristic of clinical influenza; that illness began either at about the same time as the pneumonia or within a few days previous to the onset of pneumonia.

REFERENCES

1. Collins, S. D.: Excess Mortality From Causes Other Than Influenza and Pneumonia During Influenza Epidemics, Pub. Health Rep. **47**: 2159-2179, 1933.
2. Pearson, H. E., Eppinger, E. C., Dingle, J. H., and Enders, J. F.: A Study of Influenza in Boston During the Winter of 1940-1941, New England J. Med. **225**: 763-770, 1941.
3. Finland, M., Peterson, O. L., and Strauss, E.: Staphylococcic Pneumonia Occurring During an Epidemic of Influenza, Arch. Int. Med. **70**: 183-205, 1942.
4. Wollenman, O. J., Jr., and Finland, M.: Pathology of Staphylococcal Pneumonia Complicating Clinical Influenza, Am. J. Path. **19**: 23-41, 1943.
5. Finland, M., Barnes, M. W., and Samper, B. A.: Influenza Virus Isolations and Serological Studies Made in Boston During the Winter of 1943-1944, J. Clin. Investigation **24**: 192-208, 1945.
6. Parker, F., Jr., Jolliffe, L. S., Barnes, M. W., and Finland, M.: Pathologic Findings in the Lungs of Five Cases From Which Influenza Virus Was Isolated, Am. J. Path. **22**: 797-819, 1946.
7. Stuart-Harris, C. H., Andrewes, C. H., and Smith, W.: A Study of Epidemic Influenza: With Special Reference to the 1936-7 Epidemic, Medical Research Council, Special Report Series No. 228, His Majesty's Stationery Office, London, 1938.
8. Stokes, J., Jr., and Wolman, I. J.: Probable Synergism of Human Influenza Virus and Staphylococcus aureus in Rapidly Fatal Respiratory Infection, Internat. Clin. **1**: 115-122, 1940.
9. Scadding, J. G.: Lung Changes in Influenza, Quart. J. Med., N. S. **6**: 425-465, 1937.
10. Nigg, C., Eklund, C. M., Wilson, D. E., and Crowley, J. H.: Study of an Epidemic of Influenza B, Am. J. Hyg. **35**: 265-284, 1942.
11. Commission on Acute Respiratory Diseases and New York State Department of Health: The Relation Between Epidemics of Acute Bacterial Pneumonia and Influenza, Science **102**: 561-563, 1945.
12. Himmelweit, F.: Influenza Virus B Isolated From a Fatal Case of Pneumonia, Lancet **2**: 793-794, 1943.
13. Jackson, W. P. U.: Influenza B Among West Indians: Outbreaks in Bahamas and in England, Lancet **2**: 631-635, 1946.
14. Burnet, F. M., Stone, J. D., and Anderson, S. G.: An Epidemic of Influenza B in Australia, Lancet **1**: 807-811, 1946.
15. Dudgeon, J. A., and others: Influenza B in 1945-1946, Lancet **2**: 627-631, 1946.
16. Finland, M., Barnes, M. W., Meads, M., and Ory, E. M.: Serologic Studies of Influenza Made in Boston During the Winter of 1945-46, J. Lab. & Clin. Med. **33**: 15, 1948.
17. Finland, M., Barnes, M. W., Meads, M., Ory, E. M., and Rubinstein, A. D.: Influenza B in Needham, Massachusetts, December, 1945. Serologic Studies in Cases and in Family Contacts, J. Lab. & Clin. Med. **32**: 367-378, 1947.
18. Finland, M., Peterson, O. L., Samper, B. A., and Barnes, M. W.: Cold Agglutinins. I. Occurrence of Cold Isohemagglutinins in Various Conditions, J. Clin. Investigation **24**: 451-457, 1945.

NUTRITIONAL STATUS AND INFECTION RESPONSE

I. Electrophoretic, Circulating Plasma Protein, Hematologic, Hematopoietic, and Immunologic Responses to Salmonella Typhimurium (Bacillus Aertrycke) Infection in the Protein-Deficient Rat

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INTRODUCTION

THE relationship between hunger, famine, and infection[1] has become more conspicuous by reiteration than by demonstration. Each newly isolated nutritional factor has been indicted for some inadequacy of infectious disease response.[2] In recent years, protein nutrition, in particular, has acquired considerable prominence in this regard. It has been inferred that protein inanition would increase susceptibility or decrease resistance to infectious disease by altering protective responses.[3, 4] Clinical observations[5-7] and specific laboratory investigations[8-16] have somewhat implemented this hypothesis.

In general, laboratory studies pertaining to response to infection associated with protein deficiency have measured phagocytic indices,[12, 13] humoral antibody titers produced by a nonpathogenic antigen,[10, 12, 14, 15] or a lethal end point produced by a virulent, viable pathogen.[8, 9, 11, 16] Concomitant studies of variations in specific physiologic responses to diet and infection have been rather limited.[17-19] The response of an organism to infection under any imposed condition is characterized by many adaptative phenomena. To further test the implications of protein deficiency and infection, observations of several simultaneous physiologic adaptations to controlled dietary deficiency and specific infection are necessary. To this end, data are presented indicating growth, food consumption, concentration, total circulating and electrophoretic distribution of plasma protein, and bacteriologic, immunologic, and hematologic studies in control and Salmonella-infected rats, both well nourished and protein deficient.

METHOD

After an observation period on adequate or protein-deficient diets, animals were infected with virulent *Salmonella typhimurium*. Controls with regard to diet and infection were maintained. Preliminary experiments involving sizable groups of rats were performed to determine the 50 per cent lethal dose of viable pathogen, the incidence and duration of bacteremia, the alteration in leucocyte, hemoglobin, and plasma protein concentrations associated with infection and adequate or deficient diet, and the general trend of antibody titers. These studies were done before and one, three, seven, fourteen, twenty-one, and twenty-eight days after infection. The more extensive study comprising this report was based upon the unreported data of these preliminary experiments. In this study, blood samples for the various

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determinations were drawn immediately prior to initiation of dietary deficiency, just before infection, and two, five, ten, fourteen, twenty-one, twenty-eight and forty-two days after infection. Twenty-eight days after infection all animals were injected subcutaneously with *S. typhimurium* O antigen. The immune response was determined two weeks later. Antibody titers and electrophoretic distributions of plasma proteins were measured concurrently on the twenty-eighth and forty-second postinfection days. The experiment was terminated on the forty-second postinfection day, forty-eight days after protein deficiency was started and fifty-eight days after the experiment began.

Young, growing, male, Sherman strain rats weighing 60 to 70 grams were used. In this study, four experimental groups were established as follows:

	WELL NOURISHED	PROTEIN DEFICIENT	TOTAL
Infected	20	18	
Noninfected	10	12	
	30	30	60

The animals were isolated in an air-conditioned room and kept in individual wire cages on wide meshed wire bottoms. The diets used had the following composition:

DIET COMPOSITION PER 100 GM. RATION

FOOD	ADEQUATE RATION	PROTEIN-DEFICIENT RATION
Casein	18 Gm.	2 Gm.
Sucrose	73 Gm.	89 Gm.
Mazola	5 Gm.	5 Gm.
Phillips and Hart salts IV[20]	4 Gm.	4 Gm.
Cystine	0.2 Gm.	0.2 Gm.
Thiamine chloride	200 gamma	400 gamma
Pyridoxine	200 gamma	400 gamma
Riboflavin	400 gamma	800 gamma
Niacin	2.5 mg.	5.0 mg.
Calcium pantothenate	1.5 mg.	3.0 mg.
Choline chloride	100 mg.	200 mg.
Inositol	100 mg.	200 mg.
Biotin	50 gamma	100 gamma

Haliver oil was fed in 0.1 c.c. amounts biweekly; water was offered ad libitum. Diets were isocaloric, providing 4.1 calories per gram ration. The 18 per cent casein diet afforded 155 to 368 mg. nitrogen per twenty-four hours, depending upon consumption; the 2 per cent casein diet, 19 to 39 mg. nitrogen per twenty-four hours. Twenty-four hour food consumption was recorded biweekly.

The stock culture of the infecting organism, *S. typhimurium (Bacillus aertrycke)*, was originally isolated from a mouse epizootic and subsequently maintained in a frozen state on an agar slant.* This organism was characterized by smooth, pearly white, small, elevated colonies which produced acid and gas with mannite, dulcite, maltose, dextrose, arabinose, inositol, sorbitol, and rhamnose and in Russell double sugar media; lead acetate was reduced; no reaction occurred with lactose, sucrose, or xylose. Subcultures at 37° C. in tryptic digest broth were made sequentially at twenty-four, twenty-four, eighteen, and six hours. Diluted aliquots of the final, young, six-hour subcultures were injected. In the preliminary studies a 10^{-3} (1:1,000) dilution was found to provide a 50 per cent lethal intraperitoneal inoculum. In this experiment, 1 ml. of a similar dilution containing 300,000 viable cells per milliliter was used in an endeavor to provide a 100 per cent infection rate without subsequent mortality.

At previously noted intervals, 1 ml. of blood was obtained by heart puncture under light ether anesthesia. Sterile technique was used. An initial 0.1 ml. of the sterile sample was placed in blood culture tubes containing 5 ml. freshly prepared tryptic digest broth. The remainder of the sample, placed in small tubes containing 0.2 mg. dried heparin, sufficed for all except electrophoretic determinations.

*We are indebted to Dr. John Enders for the stock culture used.

Blood cultures were incubated for twenty-four hours at 37° C. Positive growths were plated out on eosin-methylene blue and blood agar plates; the small violet and small pearly white colonies, respectively, of gram-negative bacilli were appropriately identified. All negative cultures were kept five to seven days before being discarded.

Hemoglobin (oxyhemoglobin) was determined in the Klett-Summerson photoelectric colorimeter, using a 540 mμ filter, and the relative cell volume was calculated from it.[21] Total plasma protein concentration was determined gravimetrically by the copper sulfate method, using the formula: Total protein (gm./100 c.c.) = 389.6 (Gp − 1.0079).[22] Total leucocyte and differential counts were done by the usual methods. Blood volume partition studies, using Evan's blue dye T-1824 and a modified single sample photoelectric microcolorimetric technique,[21] were done concurrently with the concentration determinations at several intervals. Total circulating hemoglobin and total circulating protein were readily calculated and adjusted to unit values (grams per unit blood or plasma volume). These unit values were derived by adjusting total volumes to a unit of surface area (milliliters per 100 cm.^{2}).[23] Surface area was calculated according to the formula: SA (cm.^{2}) = 12.54 (weight)$^{0.60}$.[24]

Humoral group and type-specific antibody titers were determined by a standard two-fold, eight-tube serum dilution agglutination method. Five-tenths milliliter of a 1:10 plasma mixture and 0.5 ml. of the appropriate antigen were used in the first tube. The formalin-treated, diluted suspension for floccular agglutination (H or flagellar antigen) and the alcohol-treated, diluted suspension for granular agglutination (O or somatic antigen) were prepared from the original *S. typhimurium* inoculum by standard Massachusetts State Laboratory procedures.

Several animals of each group were sacrificed by heart puncture on the twenty-eighth postinfection day, at the time of subcutaneous injection of O antigen in the remaining rats. Sera from the sacrificed animals were pooled by group for electrophoretic analyses. Electrophoreses of the sera were carried out in a modified Tiselius apparatus.* Three to five milliliters of serum were diluted to 12 ml., giving approximately 2 per cent protein concentration, and subsequently dialyzed against sodium diethyl barbiturate buffer of pH 8.6 and 0.1 ionic strength. A 10 ml. cell was used and runs of 7,200 seconds were made at 1 to 2° C. and potential gradients of 5 to 6 V per centimeter. Photographs of schlieren patterns, obtained with a cylindrical lens at an angle of 45°, were made and enlarged 1:5 by projection. Mobilities were calculated from descending boundary patterns. Relative proportions of the various plasma components were determined by planimetry of Gauss-curve resolved areas. The reported compositional data represent an average derived from both ascending and descending boundary patterns, excluding the delta and epsilon boundary anomalies.

At time of death, bone marrow imprints[26] were obtained from the distal diaphysial third of the left femur; eight to ten consecutive imprints were made on each of three cover slips. A modified May-Grünwald-Giemsa stain was used. Each of one hundred adjacent cells derived from a well-defined peripheral area of five consecutive imprints was identified and tabulated. Cytologic classification followed that of Stasney and Higgins,[26] but megakaryocyte, myeloblast, promyelocyte, myelocyte, metamyelocyte, granulocyte, lymphoblast, lymphocyte, plasma cell, reticulum cell, erythroblast, pronormoblast, normoblast, and unclassified cells were enumerated. To simplify evaluation, these were grouped as immature myeloid forms, mature granulocytes, erythroblasts, normoblasts, lymphocytes, and other miscellaneous cells. Total marrow cellularity was not estimated.

RESULTS

Results are summarized in Tables I through VI and Figs. 1 through 3.

DISCUSSION

Growth.—

Caloric and Nitrogen Consumption: Weight increments of 3 to 5 Gm. per day on the initial control diet represented an adequate growth rate for rats of

*According to the technique of the Department of Physical Chemistry.[25]

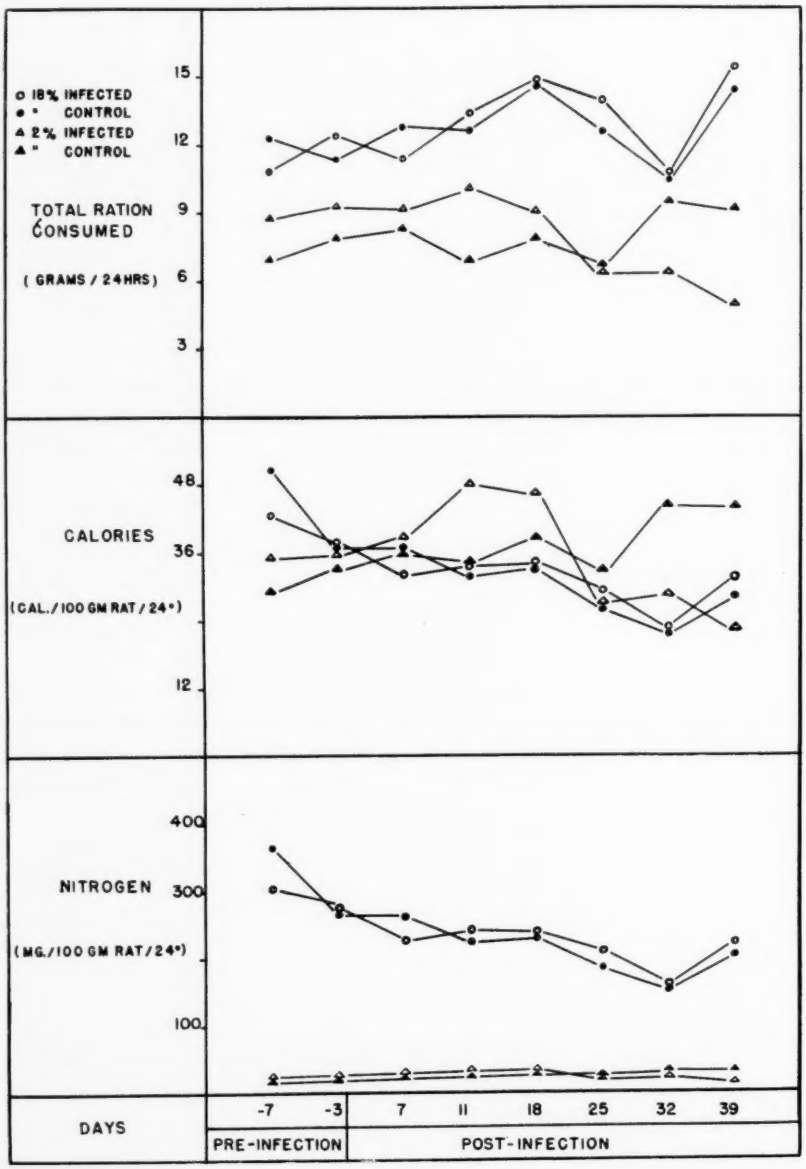

Fig. 1.—Relative average food consumption of control and infected rats on 18 per cent and 2 per cent protein diets. Total ration consumed, as measured over a twenty-four hour period, is compared with caloric and nitrogen intakes adjusted to a unit of body size.

this age, sex, and strain, and for this diet. Progressive weight loss, ultimately amounting to 20 to 25 per cent of the starting body weight, attended protein deficiency. Growth responses to the 18 per cent and 2 per cent casein diets are indicated in Fig. 1. Total food consumption per twenty-four hours per rat was greater in the well-nourished than in the deficient animals (10 to 15 Gm. per twenty-four hours versus 6 to 10 Gm. per twenty-four hours). The relative

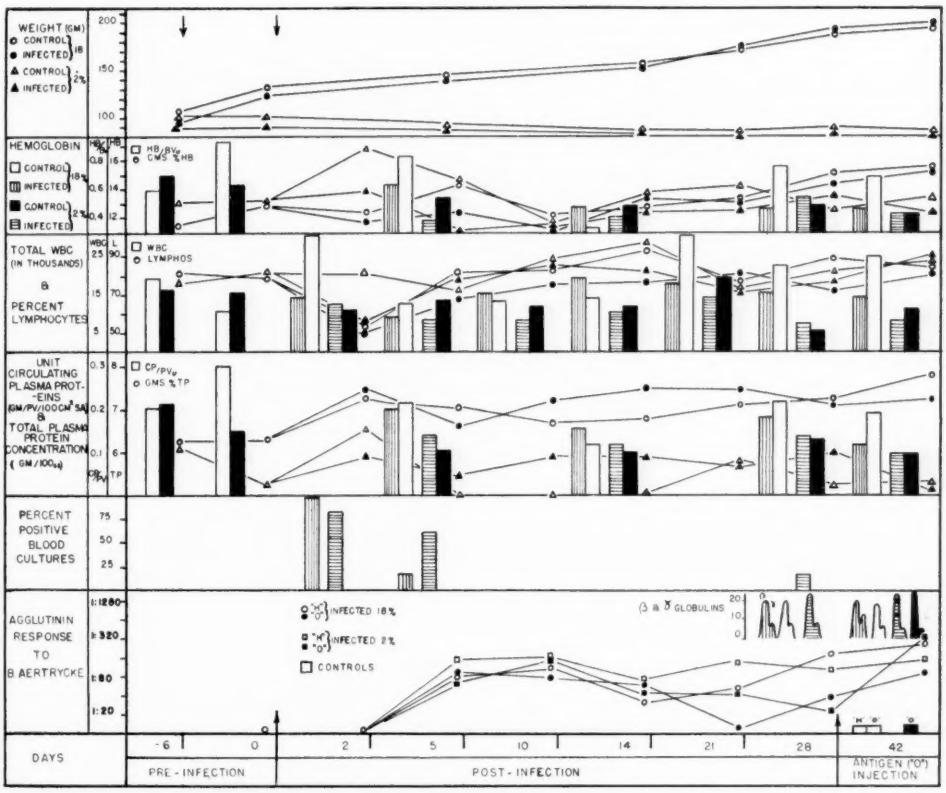

Fig. 2.—Summary of the blood and bacteriologic findings in control or Salmonella-infected rats on 18 per cent or 2 per cent protein rations.

caloric consumption per 100 Gm. rat per twenty-four hours, however, was similar in both groups. During some phases of the study, the protein-deficient animals appeared to consume more calories per 100 Gm. rat than did the well-nourished animals. Irrespective of infection, well-nourished animals consumed 155 to 368 mg. N per 100 Gm. rat per twenty-four hours, and deficient animals, 19 to 39 mg. N per 100 Gm. rat per twenty-four hours (Fig. 1). The similarity of nitrogen consumption in the infected and noninfected animals and the lack of striking differences (irrespective of dietary protein level) in caloric intakes per 100 Gm. rat per twenty-four hours serve in lieu of pair-fed inanition control data. Nitrogen consumption of the animals receiving an adequate diet appeared to be inversely proportional to growth and unaltered by infection. Nitrogen consumption of the deficient animals was maintained at a relatively constant level despite both infection and progressive weight loss.

Blood Studies.—

Hemoglobin: Hemoglobin concentration values in grams per 100 ml. for rats of this age, strain, and sex range from 12.20 ± 0.9 to 15.1 ± 0.3[22] and are similar to values reported for other strains.[27] Hemoglobin concentrations of infected and noninfected animals followed similar trends. Differences between

TABLE I. AVERAGE CONCENTRATION VARIATIONS

	PREINFECTION PERIOD			DURATION OF									
	INITIAL (10 DAYS)	DEFICIENCY (6 DAYS)		48 HOURS				5 DAYS				10	
Dietary level	18%	18%	2%	18%		2%		18%		2%		18%	
Infectious status	C.	C.	C.	I.	C.	I.	C.	I.	C.	I.	C.	I.	C.
Number of animals†	6	3	3	5	2	5	2	5	2	5	2	5	2
Hemoglobin (Gm./100 c.c.)	12.4	13.1 (2)‡	13.1	11.9	12.8 (1)	14.0	17.0	12.8 (3)	14.4	10.6	14.7	11.0	12.3
Total W.B.C./mm.³ (in thousands)	17.1	11.2	15.5	14.7	36.4 (1)	12.3	11.5	9.1 (4)	12.4	8.8	13.6	15.2	13.2
Per cent polymorphonuclear leucocytes	19	18	16	48	39 (1)	44	16	29	20	17	26	21	15
Per cent lymphocytes	81	81	83	50	53 (1)	54	83	69	83	82	73	77	84
Total plasma protein (Gm. 100 c.c.)	6.19	6.26	5.32	7.40	7.24	5.85	6.43	6.73	7.05	5.57	4.98	7.19	6.74

C, control; I, infected.

*These data represent a series of longitudinal determinations within specific groups. They involved re

†Number of animals contributing to each mean is small. Standard deviation of hemoglobin and total pro 0.95–1.29.[23]

‡Numbers in parentheses represent number of animals in group when that figure differs from number in

TABLE II. AVERAGE CIRCULATING PROTEIN AN

	PREINFECTION PERIOD						
	INITIAL (10 DAYS)	DEFICIENCY (6 DAYS)		5 DAYS			
Dietary level	18%	18%	2%	18%		2%	
Infectious status	0	0	0	I.	C.	I.	C.
Number of animals†	6	3	3	2	2	2	2
Surface area (S.A. = cm.²)	203.5	225.0	192.0	270.9	265.1	182.4	171.8
Plasma protein concentration (TP = Gm./100 c.c.)	6.18	6.26	5.29	6.69	7.05	5.43	4.49
Total plasma volume (PV_t = c.c.)	6.81	10.08	5.63	8.34	8.98	4.63	3.71
Unit plasma volume (PV_u = c.c./100 cm.² S.A.)	3.32	4.44	2.94	3.04	3.30	2.55	2.16
Total circulating protein (CP_t = Gm./PV_t)	0.42	0.64	0.29	0.55	0.64	0.25	0.19
Unit circulating protein (CP_u = Gm./PV_u)	0.21	0.29	0.15	0.20	0.23	0.14	0.11
Total blood volume (BV_t = c.c.)	10.72	16.40	9.19	13.40	15.50	6.74	6.37
Unit blood volume (BV_u = c.c./100 cm.² S.A.)	5.37	7.29	4.79	4.95	5.85	3.70	3.71
Unit circulating hemoglobin (CHb_u = Gm./BV_u)	0.67	0.95	0.63	0.63	0.84	0.39	0.35

I, infected; C, control.

*In general, each animal was subjected to only one plasma volume study; hence the data of

†The number of animals contributing to each mean is small; the standard deviation, as pre PV_t, ± 0.67–1.87; PV_u, ± 0.38–0.46; CP_u, ± 0.02–0.03; BV_t, ± 1.07–2.48; BV_u, ± 0.51–0.57; CHB_u

OF BLOOD CELLS AND PLASMA PROTEIN*

INFECTION AND DIET																	
DAYS	14 DAYS						21 DAYS				28 DAYS				42 DAYS			
	2%		18%		2%		18%		2%		18%		2%		18%		2%	
	I.	C.	I.	C.	I.	C.	I.	C.	I.	C.	I.	C.	I.	C.	I.	C.	I.	C.
	5	2	5	2	5	2	5	2	5	2	9	2	5	4	10	5	8	5
	11.0	11.3	13.5	12.9	12.9	13.8	13.2	13.3	12.7	14.2	14.5	15.2	13.8	12.7	15.4	15.5	12.6	13.6
	8.3	12.0	19.5	14.2	10.3	12.0	18.3	37.9	14.7	19.5	15.9 (8)	23.4	6.0 (4)	4.9	14.8	25.2	7.4	11.8
	11	10	20	7	16	4	17	20	20	23	25	10	19	17	18	11	11	12
	89	88	79	94	83	96	81	79	78	78	73	90	79	82	81	85	87	85
	5.97	4.94	7.30	6.85	5.96	4.94	7.48	7.14	5.63	5.68	7.10	7.24	6.51	5.82	7.61	7.82	5.08	5.45

peated observations frequently derived from the same animal for the duration of the experiment.
tein as previously determined in statistically adequate series of similar rats was: TP, ± 0.34–0.41; Hb., ±
dicated at top of column.

HEMOGLOBIN VARIATIONS WITH DIET AND INFECTION*

DURATION OF INFECTION												
14 DAYS				28 DAYS				42 DAYS				
18%		2%		18%		2%		18%		2%		
I.	C.	I.	C.	I.	C.	I.	C.	I.	C.	I.	C.	
2	2	2	2	2	2	2	2	4	4	4	4	
259.3	240.4	183.0	176.4	279.7	315.4	205.7	158.8	299.0	306.3	172.4	173.1	
7.47	6.85	5.88	4.94	7.14	7.24	6.01	5.28	7.28	7.75	5.15	5.23	
5.45	4.19	3.58	3.71	5.18	8.79	4.86	3.83	4.82	7.42	3.69	3.28	
2.11	1.75	1.92	2.11	1.84	2.96	2.36	2.43	1.62	2.40	2.12	1.99	
0.41	0.29	0.21	0.18	0.37	0.67	0.29	0.20	0.38	0.57	0.19	0.17	
0.16	0.12	0.12	0.10	0.18	0.22	0.14	0.13	0.12	0.19	0.11	0.11	
9.05	6.81	5.79	6.25	9.07	15.90	8.19	6.13	9.00	13.7	5.87	5.47	
3.49	2.57	3.16	3.55	3.26	5.05	3.98	3.86	3.01	4.47	3.41	3.16	
0.47	0.33	0.41	0.49	0.47	0.76	0.55	0.49	0.46	0.69	0.43	0.43	

this table represent determinations on approximately fifty rats.
ously determined in statistically adequate groups of similar rats, was as follows: TP, ± 0.34–0.41;
0.09–0.10.[23]

well-nourished and deficient animals were apparent only shortly after infection was initiated and again forty-two days later as the experiment was concluded. The immediate postinfection period was accompanied by higher hemoglobin concentrations in the infected and control protein-deficient animals (Table I). Hemoconcentration attending this phase of acute protein deficiency would seem to account for the high levels noted and serve to mask a suggestive depletion of unit circulating hemoglobin in the protein deficient groups[28, 29] (Table II, Fig. 2). However, a consistent relationship between hemoglobin concentration and unit circulating hemoglobin was not demonstrable during this early phase of rapid adjustment to infection and diet. At twenty-eight and forty-two days after infection, both hemoglobin concentrations and unit circulating hemoglobin levels appeared to be diminished in the protein-deficient animals, without significant difference being imposed by infection. This finding would seem compatible with the observed anemia associated with long-continued low casein diets[28-31] and is possibly related to impaired globin formation. The decreased unit circulating hemoglobin of the well-nourished infected animals was masked by the decreased plasma volume and resultant apparent hemoglobin concentration. With the exception of a probable technical error on the fourteenth postinfection day, the circulating hemoglobin of the uninfected, well-nourished rats was somewhat greater than that noted in the other groups. The relative anemia of the well-nourished infected animals may represent an anemia of infection, presumably related to hypoferremia.[32]

Total Leucocyte and Differential Counts: Reported values for total leucocyte and differential counts in rats approximate 8 to 15,000 white blood cells per cubic millimeter, of which 4 to 25 per cent are polymorphonuclear granulocytes; the remainder, largely lymphocytes.[27] Infected and control animals on the 2 per cent protein diet manifested an absolute leucopenia after approximately three weeks of dietary deficiency. Leucopenia[13] and granulocytopenia[33] have been observed to attend protracted dietary protein restriction. Well-nourished uninfected rats appeared to have the highest total white blood cell counts after the second post-infection week. No other striking differences in total white blood cell counts of the various experimental groups were evident. Relative lymphopenia was observed in both groups of infected animals forty-eight hours after infection was initiated. The 18 per cent control value inadvertently represented a single animal observation. With this possible exception, the immediate postinfection lymphopenia would seem to be in accord with the alarm reaction phase of the general adaptation syndrome of Selye[34, 35] and may have represented a period of rapid lymphocyte dissolution with attendant elaboration of antibody globulin[36, 37] in both groups of infected rats, although no antibody titers were demonstrable at that time.

Hematopoiesis: Peripheral blood leucocytes and hemoglobin variations were associated with suggestive hematopoietic changes in the femoral bone marrow. The distribution of cellular elements in the femoral marrow of the twenty-seven well-nourished and twenty-five deficient rats examined was similar to that reported in twenty-four Wistar strain[26] and twelve Rockland strain rats.[38] Protracted protein deficiency was associated with a tendency toward a relative

TABLE III. AVERAGE VARIATIONS IN FEMORAL BONE MARROW CYTOLOGY WITH INFECTION AND DIET

GROUP	NUMBER OF ANIMALS	RANGE AND AVERAGE VALUES (%) PER 500 CELLS COUNTED* IMMATURE MYELOID FORMS	MATURE GRANULOCYTES	ERYTHROBLASTS	NORMOBLASTS	LYMPHOCYTES†	MISCELLANEOUS	MYELOID	ERYTHROID	M:E
28 days postinfection										
18% Infected	9	22.8-46.2 30.2	15.6-31.6 20.2	2.0-16.8 8.7	27.0-51.0 34.8	0.2-7.4 3.7	0.8-6.0 1.8	50.4	43.5	1.16
18% Control	2	29.7-39.1 35.4	9.7-23.8 16.8	6.5-9.7 8.1	27.2-40.0 33.6	2.7-7.3 5.0	0.8-1.4 1.1	42.2	41.7	1.26
2% Infected	4	35.3-42.3 39.2	18.1-31.0 21.9	2.0-10.6 5.2	21.1-40.0 29.1	1.5-7.7 3.6	1.0-2.0 1.4	61.1	34.3	1.78
2% Control	4	27.9-60.7 43.1	3.0-36.4 18.2	3.6-14.1 8.4	22.8-31.2 26.8	0.8-2.8 1.6	1.0-5.0 2.1	61.3	34.9	1.76
42 days postinfection										
18% Infected	9	10.8-35.8 22.9	18.4-40.4 26.9	1.2-14.4 4.0	33.2-55.5 42.0	0.6 0.6	2.6-7.0 3.9	49.8	46.0	1.08
18% Control	7	20.0-42.6 28.5	18.4-30.8 26.0	2.4-18.2 7.5	16.8-44.2 34.3	0.6-2.4 1.4	1.8-7.0 3.1	54.5	41.8	1.30
2% Infected	9	15.4-44.4 29.7	17.8-34.4 26.2	2.8-27.0 9.8	18.2-46.2 30.1	0.2-4.7 1.5	1.6-4.2 3.0	55.9	39.9	1.40
2% Control	8	18.0-47.8 32.2	18.0-32.4 26.0	1.6-8.4 5.0	18.4-46.2 32.6	0-4.4 1.6	0.8-7.6 2.7	58.2	37.6	1.55

*Cytologic differentiation in general followed that of Stasney and Higgins. We are indebted to Dr. A. Lapi and to Miss Doris Wilson for assistance with the counts. With the exception of lymphocyte counts, consistent definition was achieved. Immature myeloid forms include myeloblasts, promyelocytes, myelocytes, metamyelocytes; normoblasts include pronormoblasts; miscellaneous includes plasma cells, reticulum cells, megakaryocytes, and unclassified cells.

†The majority of the low lymphocyte counts in the forty-two day postinfection groups were recorded by one observer.

increase of immature myeloid forms without apparent increase in mature granulocytes, either in the marrow or peripheral blood. This may have been a compensatory response to peripheral leucopenia, or might suggest a granulocyte maturation inadequacy or arrest. Depletion or inhibition of some factor necessary for production of the mature granulocyte may have been responsible. The phenomenon appeared to be associated with dietary protein deficiency irrespective of infectious status. Fewer maturing erythroid forms, particularly normoblasts, were noted in the deficient rats. The diminished erythropoietic activity of the protein deficient rat may have resulted from inadequate globin formation and may have been partially responsible for the observed depletion of unit circulating hemoglobin. At the level of hemoglobin synthesis attained, hypoferremia associated with infection[32] appeared to be a less important limiting factor. The tendency of infected well-nourished animals toward a larger proportion of erythroid forms than the uninfected controls might reflect a compensatory response to relative depletion of unit circulating hemoglobin. Presumably adequate globin synthesis occurred on an 18 per cent casein diet. Accordingly, at the level of hemoglobin synthesis in the well-nourished infected rat, hypoferremia may have been a limiting factor and partially responsible for the observed depletion of unit circulating hemoglobin.

Hematopoiesis, as reflected in the femoral marrow myeloid-erythroid differences, appeared to be more influenced by diet than by infection. The immune response observed at forty-two days postinfection did not appear to be associated with distinct variations in the femoral bone marrow picture in any of the groups.

Plasma Proteins: Total plasma protein concentrations in rats of this age, sex, and strain vary from 6.60 ± 0.1 to 8.07 ± 0.07.[23, 39] In this particular experiment, the total plasma protein concentration appeared to reflect the level of protein nutrition of the animal. In general, however, the measurement of concentration provides an inconsistent indication of total circulating proteins owing to plasma volume variations.[40, 41] Protein deficiency per se is attended by significant decreases in unit circulating plasma proteins[28, 29, 42-44] (Fig. 2, Table I). Infection per se did not appear to induce significant change in total plasma protein concentrations. However, the unit circulating proteins of the infected rats on the 18 per cent protein diet were somewhat reduced after twenty-eight to forty-two days, in comparison with the appropriate controls, but exceeded those of the protein-deficient animals. Both groups of deficient animals had similar unit circulating protein levels.

Infection per se appeared to be associated with minimal change in plasma protein concentration, unit circulating protein, and electrophoretic plasma protein components. The electrophoretic distribution of plasma components, like the other protein measurements, varied with the dietary status. Fairly adequate resolution of the major plasma protein components (albumin, alpha, beta plus fibrinogen, and gamma globulins) was possible with a diethyl barbiturate buffer at pH 8.6, under the conditions of electrophoresis. More detailed differentiations of alpha-1 and alpha-2 globulins and of alpha-2 and beta globulins were less satisfactory. However, assuming six components, Gauss probability curves were con-

TABLE IV. ELECTROPHORETIC MOBILITIES OF WELL-NOURISHED AND PROTEIN-DEFICIENT RATS

	"f"	ALBUMIN	α_1	α_2	β_1	$\beta_2 + \Phi$	γ
18% Casein diet							
Range	7.6-8.0	5.7-6.3	4.7-5.3	4.1-4.6	3.4-3.8	2.5-2.6	1.4-1.5
Mean	7.9	6.0	5.0	4.3	3.6	2.6	1.4
2% Casein diet							
Range	7.1-7.7	5.5-6.0	4.3-5.0	3.2-3.6	3.7-4.2	2.2-2.6	0.9-1.5
Mean	7.3	5.9	4.8	3.5	4.1	2.5	1.3

Mobilities calculated from descending boundary patterns only, according to the formula $\triangle h \times \frac{K \ (0.75)}{T \text{ sec. (Milliamperes)}}$, hence all values represent $M \times 10^5$. The lower values in the range of 2 per cent diet plasma mobilities are contributed entirely by the 2 per cent control group after twenty-eight days of diet deficiency. This analysis was run some months after those of the seven other groups. All means are weighted by relative numbers of animals per group contributing to the value.

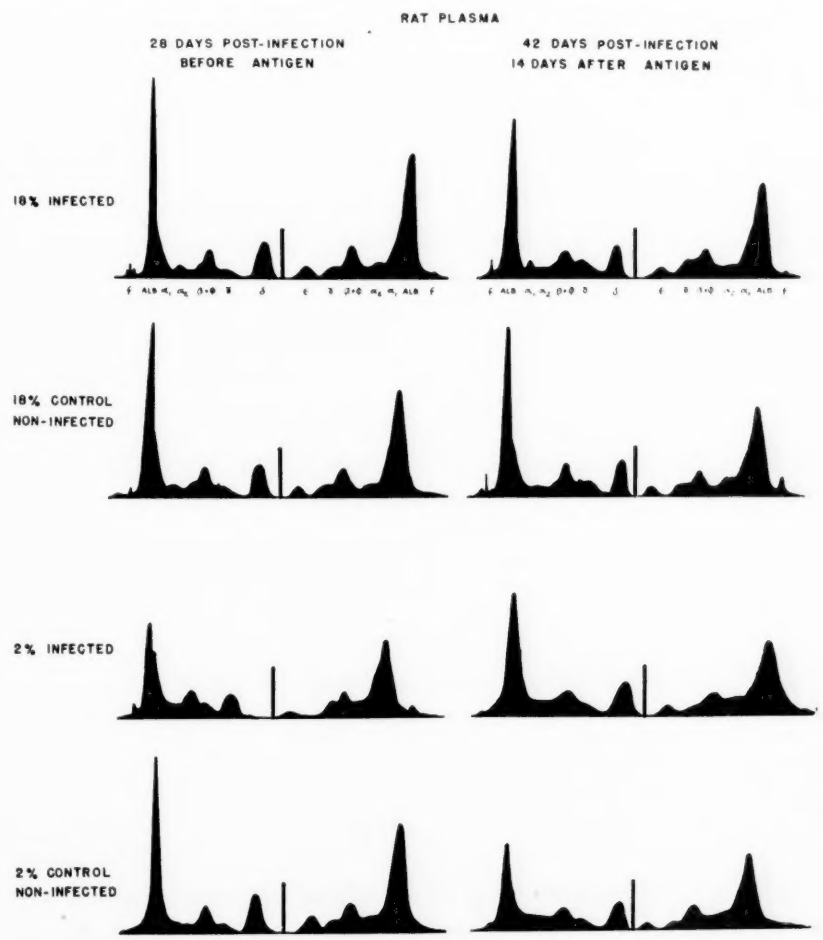

Fig. 3.—Schlieren patterns of the electrophoretic analyses of rat plasma pools derived from infected and noninfected rats on 18 per cent and 2 per cent protein rations. Both ascending and descending boundary patterns are shown twenty-eight days after infection (prior to antigenic stimulation) and fourteen days later, following the injection of *S. typhimurium* O antigen. All determinations were made using a sodium diethyl barbiturate buffer at pH 8.6 and 0.10 ionic strength. The 2 per cent diet infected preantigen pattern and the 2 per cent diet control postantigen pattern represent protein concentrations of 1.2 and 1.4 per cent respectively. The remainder of the patterns represent analyses at 2.0 ± 0.1 per cent protein concentrations.

TABLE V. ELECTROPHORETIC VARIATIONS IN

GROUP	NUMBER OF ANIMALS	PLASMA PROTEIN CONCENTRATION (GM./100 C.C.)	PER CENT PLASMA COMPOSITION*							
			"f"	ALBUMIN	α_1	α_2	β_1	$\beta_2 + \Phi$	γ	TOTAL
28 days postinfection (before second antigen)										
18% control	2	6.46	3.0	54.5	10.9	5.6	3.6	15.2	7.2	99.9
18% infected	9	6.78	2.9	51.0	11.6	7.7	4.6	15.3	7.4	99.5
2% control§	4	5.18	5.4	60.8	5.9	4.3	3.3	14.8	5.5	100.0
2% infected	4	6.28	6.9	43.2	12.0	6.6	5.7	18.0	7.7	100.1
42 days postinfection (after second antigen)										
18% control	5	7.12	4.2	57.0	6.7	7.2	3.0	15.2	6.9	100.2
18% infected	10	7.36	3.4	55.6	5.9	4.5	3.5	16.1	10.7	99.7
2% control	5	5.68	3.3	49.0	11.5	6.1	5.8	18.4	6.0	100.1
2% infected	8	5.13	8.5	48.7	9.8	5.8	5.3	16.7	5.3	100.1

*Somewhat arbitrary values derived from relative areal resolution and representing average per

†Derived from the product of the plasma protein concentration of the pool and the per cent

‡Represents the product of the average unit circulating proteins (Table II) derived from the of the resolved schlieren pattern.

§Two per cent control calculations were derived from electrophoretic runs carried out several

sistently and reasonably, if somewhat arbitrarily, drawn. Separation of beta-1 and beta-2 globulins was not entirely satisfactory in these analyses, although distinct demarcations were apparent in some instances. Beta-2 globulin and fibrinogen were not separated under the conditions of electrophoresis and, accordingly, were considered together. Gamma globulin was quite distinct in all patterns and was well separated from the boundaries. A so-called "f" component with mobility greater than that of albumin has been noted in rat plasma[45] and consistently appeared in our patterns. Its significance is not known. The mobilities of the various components (Table IV), calculated from the descending patterns, are similar to those reported for other rats[45, 46] and roughly approximate the mobilities of human plasma components.

The plasma composition is indicated in Table V. Typical patterns are illustrated in Fig. 3. Since all electrophoretic data were derived from pooled samples, the range and standard deviation of individual rat variation were not evident. The composition of rat plasma appears to vary with the strain used.[46] Previous reports have been largely concerned with the Long-Evans[46, 47] or Sprague-Dawley strains.[45] In the Sherman strain rat used in this study, the plasma composition was similar to that observed in man;[48-50] gamma globulin values were slightly lower.

Concentrations of the various plasma components were calculated from the proportionate composition of schlieren patterns and the total plasma protein concentrations of the respective pools. Since unit circulating protein data appear to be more reliable than simple concentration measurement as an indicator of plasma protein variation, the total circulating amount of each plasma component in the vascular compartment was also calculated and adjusted to unit value. Such arbitrary calculation was dependent on resolution of electrophoretic

PLASMA PROTEINS WITH INFECTION AND DIET

CONCENTRATION OF PRINCIPAL PLASMA PROTEIN COMPONENTS (GM./100 C.C.)†							UNIT CIRCULATING PLASMA PROTEIN COMPOSITION (GM./UNIT PLASMA VOLUME)‡							
"f"	ALBUMIN	α_1	α_2	β_1	$\beta_2 + \Phi$	γ	"f"	ALBUMIN	α_1	α_2	β_1	$\beta_2 + \Phi$	γ	TOTAL
0.19	3.53	0.70	0.36	0.23	0.98	0.47	0.006	0.120	0.024	0.012	0.008	0.033	0.016	0.22
0.20	3.45	0.79	0.52	0.31	1.04	0.50	0.005	0.092	0.021	0.014	0.008	0.029	0.013	0.18
0.28	3.14	0.31	0.22	0.17	1.10	0.28	0.007	0.079	0.008	0.006	0.004	0.019	0.007	0.13
0.43	2.71	0.75	0.41	0.36	1.13	0.48	0.010	0.061	0.017	0.009	0.008	0.025	0.011	0.14
0.30	4.06	0.48	0.51	0.21	1.08	0.49	0.008	0.108	0.013	0.014	0.006	0.029	0.013	0.19
0.25	4.10	0.43	0.33	0.26	1.18	0.51	0.004	0.067	0.007	0.005	0.004	0.019	0.013	0.12
0.19	2.78	0.65	0.35	0.38	1.02	0.34	0.004	0.054	0.013	0.006	0.006	0.022	0.007	0.11
0.44	2.51	0.51	0.30	0.27	0.86	0.27	0.009	0.054	0.011	0.006	0.006	0.018	0.006	0.11

cent composition of ascending and descending boundary patterns.
areal composition of the resolved schlieren pattern.
plasma protein concentration, plasma volume and surface area, and the per cent areal composition
months after the other seven determinations.

components, plasma protein concentrations, and mean unit plasma volumes (Table V). Accordingly, these data are more relative than absolute, owing to limitations in the various techniques employed. The results remain proportional, however, since the error was systematic.

Severe protein deficiency protracted for four to six weeks resulted in a considerable decrease in total plasma protein concentration and in unit circulating protein. This diminution in protein usually has been considered the result of albumin depletion.[43, 44] In the electrophoretic patterns, as noted by Bieler and co-workers,[51] nutritional hypoproteinemia did not appear to be characterized by a marked decrease in the proportionate area representing albumin. However, with calculation of the relative concentration (grams per 100 c.c.) of the various electrophoretic plasma protein components, it was evident that protein deficiency was characterized by a reduction in electrophoretic albumin, as had been noted in dogs.[52-54] Albumin depletion appeared more marked when interpreted in relation to the associated diminished plasma volume (unit circulating plasma protein composition).* The proportion of the pattern and the concentration of alpha and beta globulins appeared slightly increased in the hypoproteinemic rats. This observation is in general accord with that of Chow.[54] When adjusted to unit plasma volume, no distinct difference between circulating alpha and beta globulins in the well-nourished or deficient animals was noted. The most marked electrophoretic change induced by protein deficiency was the reduction in both concentration and unit circulating amounts of gamma globulin. This relative depletion was most evident, as might be expected, after six weeks of severe protein deficiency. No significant change in gamma globulin in

*The relatively high albumin of the noninfected rats on the twenty-eighth day of the 2 per cent protein diet remains unexplained. This run, having the slowest mobilities of the series, was made several months after the other determinations.

response to infection per se was noted in the electrophoretic patterns of either the well-nourished or deficient groups.

Bacteriologic and Immunologic Responses.—Infection following innoculation with *S. typhimurium* was corroborated by blood culture. The time incidence and duration as observed in the 114 animals comprising this and the preliminary experiments were somewhat variable in individual animals inoculated with the same initial dose of organisms. In some instances bacteremia was present at twenty-four hours; in others, it did not appear until forty-eight hours had elapsed. It was demonstrable for three to ten days. In this study, blood culture of infected rats on the 18 per cent protein ration revealed demonstrable bacteremia in all those cultured at forty-eight hours. One of five protein-deficient infected animals did not have a positive blood culture at forty-eight hours. At four days one of five well-nourished and three of five deficient rats had positive blood cultures. The presence of bacteremia at twenty-eight days postinfection in a single protein-deficient animal remains unexplained. (See Fig. 2.) No cultures of the noninfected control rats were positive (Table VI).

TABLE VI. BACTERIOLOGIC AND IMMUNOLOGIC MANIFESTATIONS

DIET PROTEIN LEVEL	POSITIVE BLOOD CULTURE				AVERAGE AGGLUTININ TITER*							
	18%		2%		18%				2%			
					INFECTED		CONTROL		INFECTED		CONTROL	
INFECTIOUS STATUS	INFECTED	CONTROL	INFECTED	CONTROL	H†	O‡	H	O	H	O	H	O
Number of animals	5	2	5	2	5	5	2	2	5	5	2	2
Preinfection control period	–	0 (3)§	–	0 (3)	–	–	0 (6)	0 (6)	–	–	0 (6)	0 (6)
Postinfection												
48 hours	5	0	4	0	0	0	0	0	0	0	–	–
5 days	1	0	3	0	66 (3)	90 (4)	0 (1)	0	133 (3)	80	0	0
10 days	0	0	0	0	120 (4)	80	0	0	180 (4)	152	0	0
14 days	0	0	0	0	40	88	0 (1)	0	88	64	–	0
21 days	0	0	0	0	60	4	0	0	208	144	0	0
28 days	0 (9)	0	1	0	276 (9)	44 (9)	0	0	232	44	0 (4)	0 (4)
42 days‖	0 (10)	0 (6)	0 (9)	0 (5)	400 (10)	200 (10)	4 (5)	4 (5)	356	328	0 (5)	4 (5)

*On twofold dilution basis: first tube, 1:20; second, 1:40; etc. Titers are reported as the reciprocal of the average titer of the group.

†H indicates flagellar antigen agglutination.

‡O indicates somatic antigen agglutination.

§Number in parentheses indicates number of animals represented in determination when that figure differs from number indicated at top of column.

‖Forty-second day titers represent determinations made fourteen days after injection of O antigen subcutaneously in all animals on the twenty-eighth postinfection day.

No very marked difference in the incidence of bacteremia was noted in the respective dietary groups. Two spontaneous deaths occurred during the postinfection phase, both were in protein-deficient rats. Only one was an infected animal and it died on the ninth postinfection and fifteenth diet deficient day. The other, noninfected, died on the twenty-seventh day of dietary deficiency. In preliminary experiments, an approximate 50 per cent mortality was observed in both diet groups. The majority of those spontaneous deaths occurred before the third postinfection day in the deficient rats. In the well-nourished rats,

sporadic deaths were noted over a ten-day period. The low mortality of this study would appear to be the result of the relatively small inoculum of viable cells. The host[16] and pathogen[55] variation from experiment to experiment cannot be adequately evaluated. The route of infection appears to be of considerable importance in relation to the subsequent clinical course. In per os infections with *S. typimurium* demonstrable bacteremia rarely occurs. Extension of infection is largely lymphatic. Diarrhea may characterize the clinical course. With intraperitoneal inoculation, dissemination of organisms is apparently largely hematogenous and diarrhea is not a constant finding.[56]

The average agglutinin titer to somatic and flagellar antigens is tabulated according to the reciprocal of the average titer of the group (Table VI). The trend of antibody response to infection is depicted in Fig. 2. It is evident that both O and H agglutinin titers were demonstrable at five days postinfection in both deficient and well-nourished rats. The highest recorded titer in both groups at that time was 1:160. Titers remained relatively constant, averaging approximately 1:160 for twenty-one days after infection. H agglutinin titers in both deficient and well-nourished animals were somewhat higher than O agglutinins. The difference was more marked at twenty-eight days, flagellar agglutinins in some instances attaining titers of 1:640 (seven tubes) in both diet groups. These data suggest that severe protracted protein deficiency per se did not impair circulating humoral antibody response to the virulent infecting pathogen used in these studies. Lack of significant difference in quantitative circulating gamma globulins in the two diet groups appears consistent with the observed antibody responses at twenty-eight days after infection.

On the twenty-eighth postinfection day all animals were inoculated subcutaneously with O antigen in order to test the immune and anamnestic responses. The average response of the deficient infected animals, as measured by O and H agglutinin titers, was slightly greater than that of the well-nourished animals. The highest titers were 1:640 and 1:1,280. The increase in O agglutinin titers, corresponding to an immune response, was more marked than the associated rise in H titers. The apparent immune and anamnestic agglutinin responses did not appear to correlate with the changes in quantitative circulating gamma globulin in either group. Unit circulating gamma globulin of the 18 per cent protein rats remained essentially unchanged; that of the 2 per cent protein rats was distinctly diminished, despite increased agglutinin titers in both groups. No significant differences in circulating gamma globulin were noted between infected and control animals of respective diet groups, although the control groups had negligible or no agglutinin titers at either twenty-eight or forty-two days. These data might be consistent with observations that the O antibody to another bacterial species, *Eberthella typhosa*, is not contained in the gamma globulin fraction[57] or that all gamma globulin is not antibody.[58] In this regard, it is interesting that the somatic antibody is probably more closely related to protection against infection than is the flagellar anibody.[59] The secondary rise in O titer following inoculation of type-specific O antigen in the hypoproteinemic rats would seem to indicate that dietary protein deficiency does not seriously impair the mechanism for protective antibody fabrication in

the rat. This finding is at variance with that reported by Cannon and co-workers[14, 15] and Berry and associates[12] in rats, and by Krebs[60] in one patient. It is in accord with the conclusions reached by Bieler and co-workers[51] with several human subjects with hypoproteinemia.

COMMENT

Protein deficiency causing inadequate response to infection would appear to be an oversimplification of a many-faceted phenomenon. An appropriate experimental procedure involving the single variable of protein deficiency has not yet been reported. The genetic status of the host[16] and pathogen,[56] host factors initiating antibacterial immunity,[59] the source of protein,[8] protein deficiency conditioning other nutritional inadequacies as for example that of niacin,[61] and numerous other fundamental variables have not been simultaneously controlled. Endocrine-protein metabolic interrelationships[36] and coincident nitrogen balance, liver protein, and protein synthesis studies would be helpful in interpreting the place of protein in infection response.[62]

This study represents an attempt to correlate a few related phenomena. Data derived from this and the preliminary studies manifest some degree of reproducible consistency; however, conditions attending the observations necessitate some degree of statistical inadequacy. The most satisfactory absolute measures of experimental infection with a virulent pathogen are noninfection or death—complete resistance or complete susceptibility—as specific unequivocal end points. However, physiologic variations associated with such an infection must be measured in the mid-ground between complete resistance and susceptibility, and hence are dependent upon continuity and interrupted by death. The data of this study, therefore, are not strictly comparable to those of Schneider and Webster,[16] Sako,[11] or Watson[8]; their data were concerned with unequivocal susceptibility or resistance, this study with neither. Nor is it comparable with the data of Cannon and co-workers[14, 15] or Berry and associates,[12] since in their studies infection was not initiated.

The response to specific Salmonella infection of the protein deficient rat, as demonstrated by Zilva,[63] Lassen,[56] and Guggenheim and Buechler[13] and somewhat extended by this report, is apparently not markedly different from that of the well-nourished control. Physiologic variations in circulating protein observed in the Salmonella-infected protein-deficient rats of this experiment appear to be adequately explained by the protein deficiency per se and were comparatively uninfluenced by infection. Hematologic and hematopoietic responses, on the other hand, appear to be influenced to some extent by both diet and infection. Bacteriologic and immunologic responses, in contrast to those of the plasma proteins, did not appear to be significantly altered by protein deficiency. It is perhaps irrelevant to indicate that the crude measures of bacteremia and survival are not the only manifestations of bacteriologic response, and antibody titer does not wholly define antibacterial immunity. The multiplicity of subtle changes involved in both protein deficiency and infection precludes such oversimplification of a possible interrelationship. Salmonella in-

fection, for example, is presumably an intracellular infection involving mononuclear cells of lymphoid follicles. Protein deficiency of the type initiated might not sufficiently damage these cells which presumably mediate both the course of infection and antibody response.

SUMMARY

Rigid conclusions cannot be drawn from a biologic study involving isolated changing responses, rather than a specific unequivocal end point. The data presented are consistent with those observed in preliminary experiments. The data on growing Sherman strain rats subjected to infection with virulent *S. typhimurium* and to either severe protracted protein deficiency at nitrogen intakes varying from 19 to 39 mg. nitrogen per 100 Gm. rat per 24 hours, or adequate nitrogen intakes approximating 155 to 368 mg. nitrogen per 100 Gm. rat per 24 hours, may be summarized as follows:

1. Quantitative circulating plasma proteins, particularly gamma globulins, in both well-nourished and protein-deficient animals appeared to be relatively uninfluenced by infection per se despite observed changes attending protein deficiency. With protracted protein deficiency, decreased unit circulating plasma proteins, apparently representing a depletion of albumin and gamma globulin components, were observed independent of infection.

2. In both well-nourished and protein-deficient rats, hematologic responses as evidenced by differential bone marrow counts, total and differential leucocyte counts, and circulating hemoglobin values appeared to be altered somewhat by both infection and diet. Decreased unit circulating hemoglobin was noted in both well nourished and deficient infected rats. The diminution was somewhat more marked in the protein-deficient animals. Total leucocyte counts of the deficient groups were somewhat lower than those of the well nourished; the initial postinfection relative lymphopenia manifested by both infected groups was similar.

3. Hematopoiesis, as reflected in the femoral marrow myeloid-erythroid differences, appeared to be influenced more by diet than by infection. Protein deficiency was associated with a tendency toward a relative increase of immature myeloid forms and decreased numbers of maturing erythroid elements. Well-nourished infected animals appeared to have a relatively greater number of maturing erythroid forms than the controls.

4. Bacteriologic and immune responses resulting from infection and secondary antigenic stimulation were essentially unaltered by severe dietary protein deficiency. No significant difference in the incidence of bacteremia was noted between infected rats on the 2 per cent or the 18 per cent casein diets. Despite relative quantitative depletion of circulating gamma globulin, protein deficient rats attained antibody titers equivalent to those of the well-nourished animals in response to infection. Following specific secondary antigenic stimulation, the rise in humoral antibody titer was more marked in the deficient than in the well-nourished rat. Despite rise in titer, no significant change in circulating gamma globulin was observed in either group.

5. In general, pertaining to the moieties tested, rats undergoing severe protein deprivation appeared to respond similarly to and as adequately as well-nourished controls to infection with *S. typhimurium*.

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REFERENCES

1. Cannon, P. R.: War, Famine and Pestilence, Scient. Monthly **56**: 5, 1943.
2. Schneider, H. A.: Nutrition and Resistance to Infection. The Strategic Situation, Vitamins & Hormones **4**: 35, 1946.
3. Cannon, P. R.: Antibodies and the Protein Reserves, J. Immunol. **44**: 107, 1942.
4. Cannon, P. R.: The Relationship of Protein Metabolism to Antibody Production and Resistance to Infection. Advances in Protein Chemistry, vol. 2, New York, 1945, Academic Press, Inc., p. 135.
5. Long, E. R.: Constitution and Related Factors in Resistance to Tuberculosis, Arch. Path. **32**: 122, 286, 1941.
6. Farr, L. E.: Significance of Protein Metabolism in the Nephrotic Child, J. Pediat. **17**: 734, 1940.
7. Coburn, A. F., and Moore, L. V.: Nutrition as a Conditioning Factor in the Rheumatic State, Am. J. Dis. Child. **65**: 744, 1943.
8. Watson, M.: Studies on the Influence of Diet on the Resistance to Infection. II. The Effect of Various Diets on the Resistance of Mice to Bacterial Infection, J. Hyg. **37**: 420, 1937.
9. Robertson, E. C. and Doyle, M. E.: Higher Resistance of Rats Fed Casein Than Those Fed Vegetable Proteins, Proc. Soc. Exper. Biol. & Med. **35**: 374, 1936-37.
10. Cannon, P. R., Wissler, R. W., Woolridge, R. L., and Benditt, E. P.: The Relationship of Protein Deficiency to Surgical Infection, Ann. Surg. **120**: 514, 1944.
11. Sako, W. S.: Resistance to Infection as Affected by Variations in the Proportions of Protein, Fat, and Carbohydrate in the Diet, J. Pediat. **20**: 475, 1942.
12. Berry, L. J., Davis, J., and Spies, T. D.: The Relationship Between Diet and the Mechanisms for Defense Against Bacterial Infections in Rats, J. Lab. & Clin. Med. **30**: 684, 1945.
13. Guggenheim, K., and Buechler, E.: Nutrition and Resistance to Infection. Bactericidal Properties and Phagocytic Activity of Peritoneal Fluid of Rats in Various States of Nutritional Deficiency, J. Immunol. **54**: 349, 1946.
14. Cannon, P. R., Chase, W. E., and Wissler, R. W.: The Relationship of the Protein-Reserves to Antibody-Production. I. The Effects of a Low Protein Diet and of Plasmapheresis Upon the Formation of Agglutinins, J. Immunol. **47**: 133, 1943.
15. Wissler, R. W., Woolridge, R. L., Steffee, C. H., Jr., and Cannon, P. R.: The Relationship of the Protein Reserves to Antibody Production. II. The Influence of Protein Repletion Upon the Production of Antibody in Hypoproteinemic Adult White Rats, J. Immunol. **52**: 267, 1946.
16. Schneider, H. A., and Webster, L. T.: Nutrition of the Host and Natural Resistance to Infection. I. The Effect of Diet on the Response of Several Genotypes of Mus musculus to Salmonella enteritidis Infection, J. Exper. Med. **81**: 359, 1945.
17. Farr, L. E., Emerson, K., Jr., and Futcher, P. H.: The Comparative Nutritive Efficiency of Intravenous Amino Acids and Dietary Protein in Children With the Nephrotic Syndrome, J. Pediat. **17**: 595, 1940.
18. Farr, L. E., McCarthy, W. C., and Francis, T., Jr.: Plasma Amino Acid Levels in Health and in Measles, Scarlet Fever, and Pneumonia, Am. J. Med. Sc. **203**: 668, 1942.
19. Grossman, C. M., Sappington, T. S., Burrows, B. A., Lavietes, P. H., and Peters, J. P.: Nitrogen Metabolism in Acute Infections, J. Clin. Investigation **24**: 523, 1945.
20. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B.: Choline in the Nutrition of Chicks, J. Biol. Chem. **138**: 459, 1941.
21. Metcoff, J., and Favour, C. B.: Determination of Blood and Plasma Volume Partitions in the Growing Rat, Am. J. Physiol. **141**: 695, 1944.
22. Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Jr., Hamilton, P. B., and Archibald, R. M.: Copper Sulfate Method for Measuring Specific Gravities of Whole Blood and Plasma, 1945, Monograph Publications, Josiah Macy Jr. Foundation.

23. Metcoff, J., and Favour, C. B.: Total Circulating Protein and Hemoglobin in the Growing Rat, Am. J. Physiol. **142**: 94, 1944.
24. Lee, M. O.: Determination of the Surface Area of the White Rat With Its Application to the Expression of Metabolic Results, Am. J. Physiol. **89**: 24, 1929.
25. Armstrong, S. H., Jr.: Conventions of Electrophoretic Analysis Used in the Control of Plasma Fractionation in the Department of Physical Chemistry, Harvard Medical School, Cambridge, 1945, Harvard University Press, p. 12.
26. Stasney, J., and Higgins, G. M.: A Quantitative Cytologic Study of the Bone Marrow of the Adult Albino Rat, Anat. Rec. **63**: 77, 1935.
27. Wintrobe, M. M.: Clinical Hematology, ed. 2, Philadelphia, Lea & Febiger, 1946.
28. Metcoff, J., Favour, C. B., and Stare, F. J.: Plasma Protein and Hemoglobin in the Protein Deficient Rat. A Three-Dimensional Study, J. Clin. Investigation **24**: 82, 1945.
29. Benditt, E. P., Straube, R. L., and Humphreys, E. M.: The Determination of Total Circulating Serum Proteins and Erythrocyte Volumes in Normal and Protein Depleted Rats, Proc. Soc. Exper. Biol. & Med. **62**: 189, 1946.
30. Orten, A. V., and Orten, J. M.: The Role of Dietary Protein in Hemoglobin Formation, J. Nutrition **26**: 21, 1943.
31. Kornberg, A., Daft, F. S., and Sebrell, W. H.: Granulocytopenia and Anemia in Rats Fed Diets of Low Casein Content, Science **103**: 646, 1946.
32. Cartwright, G. E., Lauritsen, M. A., Humphreys, S., Jones, P. J., Merrill, I. M., and Wintrobe, M. M.: The Anemia of Infection. II. The Experimental Production of Hypoferremia and Anemia in Dogs, J. Clin. Investigation **25**: 81, 1945.
33. Kornberg, A.: Amino Acids in the Production of Granulocytes in Rats, J. Biol. Chem. **164**: 203, 1946.
34. Harlow, C. M., and Selye, H.: The Blood Picture in the Alarm Reaction, Proc. Soc. Exper. Biol. & Med. **36**: 141, 1937.
35. Selye, H.: The General Adaptation Syndrome and the Diseases of Adaptation, J. Clin. Endocrinol. **6**: 117, 1946.
36. White, A., and Dougherty, T. F.: The Pituitary Adrenotrophic Hormone Control of the Rate of Release of Serum Globulins From Lymphoid Tissue, Endocrinology **36**: 207, 1945.
37. Dougherty, T. F., Chase, J. H., and White, A.: The Demonstration of Antibodies in Lymphocytes, Proc. Soc. Exper. Biol. & Med. **57**: 295, 1944.
38. Vogel, M.: The Femoral Bone Marrow Cells of the Albino Rat, Am. J. M. Sc. **213**: 456, 1947.
39. Albanese, A. A., Holt, L. E., Jr., Kajdi, C. N., and Frankston, J. E.: Observations on Tryptophane Deficiency in Rats. Chemical and Morphological Changes in the Blood, J. Biol. Chem. **148**: 299, 1943.
40. Lyons, R. C., Jacobson, S. D., and Neerkin, J. L.: Relationship Between Changes in Serum Protein Concentration and Plasma Volume in Normal Subjects, J. Lab. & Clin. Med. **30**: 404, 1945.
41. Metcoff, J., and Stare, F. J.: The Physiologic and Clinical Significance of Plasma Proteins and Protein Metabolites, New England J. Med. **236**: 26, 68, 1947.
42. Walters, J. H., Rossiter, R. J., and Lehman, H.: Blood-Volume Changes in Protein Deficiency, Lancet **252**: 244, 1947.
43. Weech, A. A., Wollstein, M., and Goettsch, E.: Nutritional Edema in the Dog. V. Development of Deficits in Erythrocytes and Hemoglobins on Diet Deficient in Protein, J. Clin. Investigation **16**: 719, 1937.
44. Madden, S. C., and Whipple, G. H.: Plasma Proteins; Their Source, Production and Utilization, Physiol. Rev. **20**: 194, 1940.
45. Deutsch, H. F., and Goodloe, M. B.: An Electrophoretic Survey of Various Animal Plasmas, J. Biol. Chem. **161**: 1, 1945.
46. Moore, D. H.: Species Differences in Serum Protein Patterns, J. Biol. Chem. **161**: 21, 1945.
47. Li, Choh Hao: Electrophoresis of Rat Sera, J. Am. Chem. Soc. **66**: 1795, 1944.
48. Cohn, E. J., Oncley, J. L., Strong, L. E., Hughes, W. L., Jr., and Armstrong, S. H., Jr.: Chemical, Clinical, and Immunological Studies on Products of Human Plasma Fractionation. I. Characterization of Protein Fractions of Human Plasma, J. Clin. Investigation **23**: 417, 1944.
49. Dole, V. P.: Electrophoretic Patterns of Normal Plasma, J. Clin. Investigation **23**: 708, 1944.
50. Longsworth, L. G., Shedlovsky, T., and MacInnes, D. A.: Electrophoretic Patterns of Normal and Pathological Human Blood Serum and Plasma, J. Exper. Med. **70**: 339, 1939.
51. Bieler, M. M., Ecker, E. E., and Spies, T. D.: Serum Proteins in Hypoproteinemia Due to Nutritional Deficiency, J. Lab. & Clin. Med. **32**: 130, 1947.
52. Zeldis, L. J., and Alling, E. L.: Plasma Protein Metabolism—Electrophoretic Studies; Restoration of Circulating Proteins Following Acute Depletion by Plasmapheresis, J. Exper. Med. **81**: 515, 1945.

53. Zeldis, L. J., Alling, E. L., McGoord, A. B., and Kulka, J. P.: Plasma Protein Metabolism—Electrophoretic Studies; Chronic Depletion of Circulating Proteins During Low Protein Feeding, J. Exper. Med. **82**: 157, 1945.
54. Chow, B. F.: The Electrophoretic Studies on the Effect of Protein Depletion on the Plasma Proteins and the Regeneration of Plasma Proteins After Oral Administration of Hydrolysates Prepared From Casein and Lactalbumin. Conference on Proteins and Protein Hydrolysates in Nutrition, New York Acad. Sc., Dec. 7, 8, 1945.
55. Schneider, H. A.: Nutrition of the Host and Natural Resistance to Infection. II. The Dietary Effect as Conditioned by the Heterogeneity of the Test Pathogen Population, J. Exper. Med. **84**: 305, 1946.
56. Lassen, H. C. A.: Paratyphoid Infections in Avitaminotic Rats, Copenhagen, 1931, Levín and Munksgaard.
57. Enders, J. F.: Concentrations of Certain Antibodies in Globulin Fractions Derived From Human Blood Plasma, J. Clin. Investigation **23**: 510, 1944.
58. Kabat, E. A.: Immunochemistry of Proteins, J. Immunol. **47**: 513, 1943.
59. Dubos, R. J.: The Bacterial Cell in Its Relation to Problems of Virulence, Immunity, and Chemotherapy, Harvard University Monograph in Medicine and Public Health No. 6, Cambridge, 1945, Harvard University Press, chap. 7, p. 236.
60. Krebs, E. G.: Depression of Gamma Globulin in Hypoproteinemia Due to Malnutrition, J. Lab. & Clin. Med. **31**: 85, 1946.
61. Krehl, W. A., Sarma, P. S., Teply, L. J., and Elvehjem, C. A.: Factors Affecting the Dietary Niacin and Trytophane Requirement of the Growing Rat, J. Nutrition **31**: 85, 1946.
62. Protein and Antibody Production, Nutrition Rev. **4**: 279, 1946.
63. Zilva, S. S.: XX. The Influence of Deficient Nutrition on the Production of Agglutinens, Complement and Amboceptor, Biochem. J. **13**: 172, 1919.

HIGH VITAMIN A INTAKE AND BLOOD LEVELS OF CHOLESTEROL, PHOSPHOLIPIDS, CAROTENE, AND VITAMINS C, A, AND E

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THE literature contains several reports relating vitamin A to the blood and tissue lipids. Josephs[1, 4] has reviewed most of the pertinent articles and from them and his own work concluded that the hypolipemia seen in a vitamin A-deficient animal is due to a specific pathologic effect of an actual internal deficiency of vitamin A. It was found that the abrupt administration of large amounts of this vitamin caused a marked rise in total serum lipids of both normal and vitamin A-deficient rats. Josephs[2, 3] suggested a simple fat solubility relationship between the vitamin and other lipids to account for this effect of vitamin A. Green,[5, 6] in studies on serum esterase and fat absorption, and Monaghan and Schmitt,[7] who investigated the effect of vitamin A upon unsaturated fatty acids, were inclined to relate vitamin A action to metabolic processes of essential cellular structures involving phospholipid complexes.

A number of workers have not been able to demonstrate the hypolipemia of vitamin A deficiency and the hyperlipemia of high vitamin A intake. Smith,[8] in fact, found an increase in blood fatty acids and cholesterol in deficient rats and Sure, Kik, and Church[9] found no change in blood fatty acids, cholesterol, and phospholipids in vitamin A-deficient albino rats. Ralli and Waterhouse[10] found an increase of blood cholesterol in deficient dogs.

If the hyperlipemia observed by Josephs in a child[4] and the hypercholesterolemia induced by vitamin A in the patients studied by Lasch[11] and Jusatz[12] are a normal response in man to large doses of the vitamin, then a study of this response may help to uncover the role of this vitamin in human biochemical processes. In addition, if this blood lipid response is a general phenomenon induced by ingestion of vitamin A, the extent of these changes needs to be determined because of the association of hyperlipemia and hypercholesterolemia with important pathologic processes.

That vitamin A may be interrelated with a number of other vitamins is clearly pointed out in the review of this subject by Moore.[13] In the present study an attempt has been made to investigate not only the relation of vitamin A intake to blood lipid levels but also to the blood levels of several of the other vitamins interrelated with vitamin A.

General Considerations.—We have had the opportunity of studying the effects of daily supplementation with 100,000 I.U. of vitamin A on a group of patients in the Western State Hospital.† Some of the effects of this supple-

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†The patients and facilities of the hospital at Steilacoom, Wash., were kindly put at our disposal by Dr. W. N. Keller for a long-term study of the effects of vitamin A supplementation. Without the assistance of Dr. William B. Dublin and Dr. Bernice M. Hazen this work would not have been possible.

mentation on the skin of these patients will be reported by Dublin and Hazen.[14] The present paper is concerned with a comparative study of the blood vitamins A, E, and C, cholesterol, and phospholipid levels in a group of patients supplemented with 100,000 units of vitamin A daily and in an unsupplemented group of patients. We have been unable to find in the literature any reports of work in which supplementation with large doses of vitamin A was continued over a long period of time and in which blood assays for these vitamins and for lipids were performed on a sufficient number of individuals to warrant a statistical treatment of the results.

The number of patients in each group at the beginning of the study was thirty-six. The patients in these groups were in similar mental and physical condition as indicated by examination and distribution curves for age, height, and weight.

The supplemented group received at all times the same institutional diet as the control group but, in addition, one capsule* daily containing 100,000 I.U. of vitamin A. Determinations† of plasma carotene, vitamin A, vitamin E, free and total cholesterol, and phospholipid as well as whole blood vitamin C were done on five occasions—once before supplementation was started and after intervals of approximately eighteen, twenty-four, and thirty-six months of supplementation. Additional determinations were made at forty-two months, six months after supplementation was discontinued. The analyses of vitamins and lipids made at the beginning of the experiment as well as the lipid analyses at the eighteen-month period have been rejected because of unsatisfactory analytical methods.

Since the blood samples were taken from the patients at Steilacoom, Wash., and a period of four to six hours necessarily elapsed before analytical work could be started in the laboratory at Astoria, Ore., the preparation and storage of blood samples will be described briefly. Fifteen to twenty milliliter samples of blood were collected from each patient of a group of fifteen to twenty-five patients approximately sixteen to eighteen hours after the last meal and sixteen to twenty-four hours after the last dose of vitamin A. Coagulation was prevented by mixing with oxalate in 30 ml. centrifuge tubes dried as described by Van Bruggen.[15] Two milliliter samples of whole blood were immediately added to 6 ml. of 6 per cent trichloracetic acid containing norit. After thorough mixing, the samples were stored on ice for transport. The plasma from the remaining blood was separated and 1 ml. was added to 24 ml. of alcohol-ether (3:1) in a 50 ml. glass-stoppered cylinder. Immediately upon arrival at Astoria the trichloracetic acid samples were filtered and the filtrates refrigerated for twenty-four to forty-eight hours until the vitamin C analyses could be made. The alcohol-ether extracts were filtered upon arrival at Astoria and analysis for cholesterol and phospholipid was begun immediately. The remaining plasma was kept on ice until the analytical work for carotene and vitamins A and E had been completed. These analyses were usually finished within forty-eight hours.

Since only twenty to twenty-five blood samples could be assayed at one time, a complete assay of the two groups required three to four weeks at intervals of 0, 18, 24, 36,

*The vitamin A capsule, known as Oleum A, is prepared by Bioproducts, Oregon Ltd., Warrenton, Oregon. Analysis shows this product to contain the following constituents, all analyses being reported as per capsule:

Weight of contents, 0.430 Gm.; free cholesterol, 2.8 mg.; total cholesterol, 8.4 mg.; phosopholipid, 0.65 mg.; vitamin E, 0.67 mg.; vitamin A, 100,000 I.U. (equivalent to 33 mg. of pure vitamin A).

†We gratefully acknowledge the assistance of Distillation Products, Inc., Rochester, N. Y., in the establishment of carotene and vitamin A and E methods. We are especially grateful to Dr. Mary L. Quaife of that organization for her practical help on this problem.

and 42 months, so that the time periods listed in this paper are given as approximately eighteen, twenty-four, thirty-six and forty-two months. To insure comparable results, an equal number of patients in each group was sampled at each assay trip.

METHODS

Petroleum ether extracts of 4 ml. of plasma were prepared for the determination of carotene, vitamin A, and vitamin E. The plasma was well mixed with 4 ml. of alcohol and then extracted once with 12 ml. of purified low-boiling petroleum ether. Extraction was done in a 50 ml. glass-stoppered cylinder by shaking for ten minutes in a horizontal position in a mechanical shaker at a speed of 160 strokes per minute with an excursion of three inches per stroke. Refrigeration of the cylinders for one hour insured good separation of the layers. Four milliliters of the petroleum ether extract were used for carotene and vitamin A determinations and 4 ml. for vitamin E.* Carotene was measured at 440 mμ and after the solvent was evaporated at 60° C. under nitrogen the vitamin A† was estimated by the Carr-Price reaction (that is, the residue was taken up in 0.5 ml. of dry chloroform, 1 drop of acetic anhydride was added, and 3.5 ml. of Carr-Price reagent were added from an all-glass apparatus that insured rapid yet accurate delivery of an anhydrous reagent). Vitamin A values were corrected for the blue color contribution of the carotene present in the sample.

Vitamin E was determined on the 4 ml. aliquot of the petroleum ether extract by the method of Quaife and Harris,[16] modified only in that smaller volumes of extract and reagents were used. The interference of vitamin A and carotinoids was eliminated by catalytic hydrogenation as described by Quaife and Biehler.[17]

Standard curves‡ for vitamin A, carotene, and vitamin E were prepared and frequently checked during the progress of the assays.

Whole blood vitamin C was determined by our semimicro modification of the method of Roe and Kuether.[18] The final total color volume was 4 milliliters.

Free and total cholesterol were determined by the digitonin method of Sperry[19] with minor modifications. The use of alcoholic solutions of digitonin as suggested by Sobel and Mayer[20] and the washing of precipitates by solutions forcibly ejected from a syringe and needle made the use of individual stirring rods unnecessary.

The lipid phosphorus fraction was obtained by evaporation of the alcohol-ether filtrate, extraction with petroleum ether, and precipitation with acetone and magnesium chloride.[21] Final phosphate determinations were made with the reagent described by Gomori.[22] The factor of 26 was used for conversion to lipid phosphorus.

Many of the various analyses were conducted in duplicate with good agreement.

RESULTS

Since the data presented below represent the results of some 1,600 determinations, space does not permit the tabulation of individual determinations. Table I gives the values of the various assays ± their standard deviation. Significance of differences in the supplemented and unsupplemented groups is indicated by "t" values.

It is apparent that although the supplemented patients received daily an amount of vitamin A at least ten or twenty times that contained in the diet of

*All colorimetric determinations were done with a Model 11 Coleman spectrophotometer. It is possible to use 4 ml. volumes in the large cuvettes of this instrument without affecting the sensitivity if the cuvette is raised, by a suitable seat in the cuvette holder, and the opening of the holder is masked to exclude the rounded portion of the cuvette and the unfilled portion of the cuvette from the path of the incident light.

†To obtain reasonably stable Carr-Price end points with the Model 11 instrument, it is necessary to reduce the intensity of the incident light to a minimum value as recommended by Caldwell and Parrish.[37]

‡Pure tocopherol, crystalline carotene (90 per cent, 10 per cent), and a distilled vitamin A concentrate containing 201,500 I. U. per gram were kindly supplied by Distillation Products, Inc., Rochester, N. Y.

TABLE I. BLOOD LEVELS OF CAROTENE, VITAMINS A, E, AND C, CHOLESTEROL, AND PHOSPHOLIPID OF INSTITUTIONAL PATIENTS, ONE-HALF OF WHOM RECEIVED A CAPSULE CONTAINING 100,000 UNITS OF VITAMIN A A DAY

	18 MONTHS				24 MONTHS				36 MONTHS				42 MONTHS*			
	N	M ± σ		t	N	M ± σ		t	N	M ± σ		t	N	M ± σ		t
Vitamin A (I.U. %)																
Control	36	149	40.9	7.95	35	185	53.4	7.1	30	135	31.0	8.0	29	123	36.7	3.2
Supplemented	35	256	68.3		33	307	84.5		25	304	101		24	156	35.7	
Carotene (μg %)																
Control	36	216	66.5	0.86	35	213	60.8	0.43	30	193	57.4	0.95	29	213	65.5	0.43
Supplemented	36	203	60.3		33	207	53.7		25	208	64.2		24	206	67.5	
Vitamin E (mg. %)																
Control	35	1.04	0.25	2.5	35	0.98	0.24	3.4	30	1.01	0.17	4.7	29	0.99	0.19	0.2
Supplemented	36	1.20	0.26		33	1.20	0.28		25	1.27	0.22		24	1.09	0.23	
Vitamin C (mg. %)																
Control					31	0.87	0.36	0.13	27	0.99	0.36	0.07	24	0.64	0.35	0.09
Supplemented					32	0.86	0.31		25	0.99	0.35		21	0.59	0.31	
Free Cholesterol (mg. %)																
Control					34	53.4	16.9	2.0	30	53.0	10.9	3.5	29	50.8	12.3	1.1
Supplemented					33	60.8	15.7		25	63.8	11.6		24	54.6	14.3	
Total Cholesterol (mg. %)																
Control					34	207	44.7	2.2	30	202	39.5	3.9	29	208	42.5	1.2
Supplemented					33	236	42.6		25	247	43.7		24	223	47.6	
Ratio F/T																
Control					34	25.6	4.4	0.83	30	26.3	3.2	0.44	29	24.3	8.8	0.04
Supplemented					33	26.4	4.1		25	26.0	2.5		24	24.4	8.2	
Phospholipid (mg. %)																
Control					34	220	63.4	1.37	30	218	32.3	2.4	29	210	50.7	0.6
Supplemented					33	243	72.7		25	246	47.9		24	219	53.9	

N, Number of patients assayed.

M, Mean of N observations ± standard deviation which is equal to $\sqrt{\frac{\Sigma (y-M)^2}{n-1}}$.

t, Fisher's[23, 24] test of the significance of differences; in practice it is equal to $\frac{D \text{ (difference of means)}}{E_D \text{ (standard error of difference)}}$. For numbers of samples between 25 and 30 a factor of 2 is assumed to be significant ($P < 0.05$).

*At thirty-six months the vitamin A given to supplemented patients was discontinued.

the unsupplemented patients, the blood level of vitamin A in the supplemented group was less than three times that in the unsupplemented group. This difference, however, was maintained throughout a period of thirty-six months of supplementation. In the six-month period when supplementation was withdrawn, the blood level of vitamin A of the supplemented group fell to a value approximating that of the unsupplemented patients.

Plasma carotene and whole blood vitamin C seem to be entirely unaffected by the supplementation, and the minor fluctuations in these two substances, seen in both groups, no doubt reflect seasonal variations in the dietary. The assays at twenty-four and thirty-six months were done in the fall and those at eighteen and forty-two months in the spring.

The small but statistically significant increases in the vitamin E level of the blood of the supplemented patients have not been reported previously. That this increase was due to the supplementation is evidenced by the abrupt return to the control level when vitamin A was withdrawn.

The supplemented patients showed a significant increase in blood cholesterol and phospholipid. At twenty-four months and thirty-six months the supplemented group was 11 per cent and 22 per cent higher in total cholesterol and 10.5 per cent and 12.8 per cent higher in phospholipid than the control group. Since these increases in cholesterol occurred in both free and total cholesterol fractions, the F/T ratio remained normal. Although the increase in cholesterol was statistically significant at twenty-four months, the phospholipid values did not become significantly different until thirty-six months. The decrease in plasma levels of both cholesterol and phospholipid of the supplemented patients, after discontinuance of supplementation, suggests a specific effect of the supplement upon the level of these lipid constituents.

DISCUSSION

Glover, Goodwin, and Morton[25] have investigated the relation of plasma vitamin A levels to those of liver stores in rats and point out that the blood level of vitamin A is proportional to the amount of free or alcohol form present in the liver and not to the total liver stores of the vitamin.[26] They suggest that the free or alcohol form of the vitamin is the "functional" form and that to supply body tissues with greater amounts of vitamin A there must be an increase in blood vitamin A alcohol. These workers found that an increase in post-absorptive free vitamin A in the blood can be obtained only by massive dosing which, by virtue of the alcohol-ester equilibrium in the liver, increases the blood vitamin A alcohol content. This fact appears to be related to the prolonged period of treatment necessary in certain skin diseases which has been reported.[27, 28]

A "sparing" or "synergistic" action of vitamin E upon vitamin A and carotene has been reported by Hickman and co-workers[29] and Harris and associates[30] and this subject has been reviewed by Moore.[13] That vitamin A has a similar effect on vitamin E is suggested by our data. Both these substances have been shown to have a similar protective effect on unsaturated fatty acids.[31] It is possible that the increased blood level of vitamin E with vitamin A sup-

samples between 24 and 36 [illegible] the vitamin A given to supplemented patients was discontinued.
*At thirty-six months the vitamin A given to supplemented patients was discontinued.

plementation may be related to an antioxidative effect of vitamin A upon the oxidative destruction of vitamin E or to decreased utilization or to both.

Under the conditions of our experiment, there appears to be a clearly significant increase in plasma cholesterol and phospholipid as a result of vitamin A supplementation. Whether these increases are a direct effect of vitamin A upon the oxidative, storage, or transport mechanisms of lipids is unknown. Chalier, Jeune, Simon, and Alacoque[32] and Wendt[33] suggested a relation of vitamin A to the thyroid gland. Recently Sadhu and Brody[34] reported that doses of 30,000 units of vitamin A a day to rats decreased thyroid size and basal metabolism. From this work they postulate a direct relationship between vitamin A, thyroid, and thyrotropic hormone. Such a relationship might account for a lowered tissue oxidation of metabolites and thus lead to an increase in blood levels of lipids. It must be kept in mind, however, that doses of this order of magnitude are close to the toxic level for rats and far exceed the nontoxic amounts used in our study.

Although the cholesterol and phospholipid levels of the supplemented patients were significantly higher than those of the unsupplemented patients, it should be pointed out that the mean values are within normal range. The relation, moreover, between the several lipids is normal. Peters and Van Slyke[35] have charted ratios for both normal and abnormal amounts of cholesterol and phospholipid. Their lipid phosphorus cholesterol ratio charted against F/T cholesterol ratios defines the limits of normal variations of these lipids. Our figures, both for supplemented and unsupplemented patients, fall within their normal area number 5. This indicates that the hyperlipemia induced by 100,000 units of vitamin A daily lies within normal limits.

The effect of supplementation for periods greater than thirty-six months cannot yet be stated with any accuracy. Preliminary treatment of data obtained from patients in the private practice of one of us (J. V. S.) who were taking 100,000 units of vitamin A daily for 5 to 10 years, indicates plasma vitamin A, vitamin E and lipid levels indistinguishable from those of patients supplemented for three years.

As far as we know, the 100,000 units of vitamin A in the capsule used in this work was the only significant added substance in the dietary intake of the patients. The cholesterol and phospholipid and oil content of the capsule was only a small increment of the daily intake of these substances and the vitamin E content was approximately 3 per cent of the estimated human daily requirement.[36]

SUMMARY

Thirty-six unsupplemented patients and thirty-six patients supplemented with 100,000 units of vitamin A daily for thirty-six months were studied. Blood assays at eighteen, twenty-four, and thirty-six months and a final assay at forty-two months, six months after supplementation was discontinued, are reported.

1. It was found that the mean plasma vitamin A level after thirty-six months of supplementation was 125 per cent higher than that of the unsupplemented patients. Six months after supplementation was discontinued, the mean levels, although still statistically higher than those of the controls, had fallen to half of their peak values.

2. No effect upon the carotene and vitamin C blood levels was observed.

3. After eighteen, twenty-four and thirty-six months the vitamin E levels of the supplemented patients were 15.4, 22.5, and 25.7 per cent, respectively, higher than control levels.

4. Both free and total plasma cholesterol levels of the supplemented patients were increased at twenty-four and thirty-six months to the extent of 13.8 and 20.8 per cent for free and 11.1 and 22.3 per cent for total cholesterol. F/T ratios, however, remained normal. Each of the increases seen in Vitamin E and cholesterol proved to be statistically significant.

5. Although the phospholipid levels of the supplemented patients were 10.4 and 18.5 per cent higher than those of the controls at twenty-four and thirty-six months, respectively, only the thirty-six month figure proved to be statistically significant.

6. Assays made six months after high vitamin A supplementation was discontinued showed that the previously indicated elevations of vitamin E, cholesterol, and phospholipid had returned to levels not statistically different from the controls.

REFERENCES

1. Josephs, H. W.: Studies in Vitamin A. Influence of Vitamin A on Serum Lipids of Normal and Deficient Rats, Bull. Johns Hopkins Hosp. **71:** 265, 1942.
2. Josephs, H. W.: Lipid Response to Vitamin A Administration. A Possible Test for Vitamin A Deficiency, Bull. Johns Hopkins Hosp. **77:** 402, 1945.
3. Josephs, H. W.: Studies in Vitamin A. Relation of Vitamin A and Carotene to Serum Lipids, Bull. Johns Hopkins Hosp. **65:** 112, 1939.
4. Josephs, H. W.: Hypervitaminosis A and Carotenemia, Am. J. Dis. Child. **67:** 33, 1944.
5. Green, H. N.: Fat Metabolism in Vitamin A Deficiency: The Blood Serum Esterase, Biochem. J. **28:** 16, 1934.
6. Green, H. N.: Fat Metabolism in Vitamin A Deficiency: The Utilization of Fat and the Desaturation of Fat in the Liver, Biochem. J. **28:** 25, 1934.
7. Monaghan, B. R., and Schmitt, F. O.: The Effects of Carotene and of Vitamin A on the Oxidation of Linoleic Acid, J. Biol. Chem. **96:** 387, 1932.
8. Smith, M. E.: The Effect of Vitamin A Deficiency on the Concentration of the Blood Lipids of Albino Rats, J. Nutrition **8:** 675, 1934.
9. Sure, B., Kik, M. C., and Church, A. E.: Effect of Vitamin A Deficiency on Concentration of Blood Lipids of Albino Rats, Proc. Soc. Exper. Biol. & Med. **30:** 620, 1933.
10. Ralli, E. P., and Waterhouse, A.: Blood Cholesterol in Dogs on an A Deficient Diet, Proc. Soc. Exper. Biol. & Med. **30:** 519, 1933.
11. Lasch, F.: Untersuchungen über die Beeinflussung des Serumcholesterins durch Vitamin A, Klin. Wchnschr. **13:** 95, 1934.
12. Jusatz, H. J.: Die Bedeutung des A-Vitamins für die Entstehung und Bekampfung von Infectionen, Deutsch. Med. Wchnschr. **60:** 573, 1934.
13. Moore, T.: Interrelation of Vitamins, Vitamins & Hormones **3:** 1, 1943.
14. Dublin, W. B., and Hazen, B. M.: To be published.
15. Van Bruggen, J. T.: A Rapid Method for Drying Oxalate Solutions, J. Lab. & Clin. Med. **31:** 717, 1946.
16. Quaife, M. L., and Harris, P. R.: The Chemical Estimation of Tocopherols in Blood Plasma, J. Biol. Chem. **156:** 499, 1944.
17. Quaife, M. L., and Biehler, R.: A Simplified Hydrogenation Technique for the Determination of Blood Plasma Tocopherols, J. Biol. Chem. **159:** 663, 1945.
18. Roe, J. H., and Kuether, C. A.: The Determination of Ascorbic Acid in Whole Blood and Urine Through the 2,4-Dinitrophenylhydrazine Derivative of Dehydroascorbic Acid, J. Biol. Chem. **147:** 399, 1943.
19. Sperry, W. M.: The Schoenheimer-Sperry Method for the Determination of Cholesterol, New York, 1943. Personal communication.
20. Sobel, A. E., and Mayer, A. M.: Improvements in the Schoenheimer-Sperry Method for the Determination of Free Cholesterol, J. Biol. Chem. **157:** 255, 1945.
21. Bloor, W. R.: The Oxidative Determination of Phospholipid (Lecithin and Cephalin) in Blood and Tissues, J. Biol. Chem. **82:** 273, 1929.

22. Gomori, G.: A Modification of the Colorimetric Phosphorus Determination for Use With the Photoelectric Colorimeter, J. Lab. & Clin. Med. **27**: 955, 1942.
23. Fisher, R. A.: Statistical Methods for Research Workers, Edinburgh, 1934, Oliver & Boyd.
24. Patterson, D. D.: Statistical Technique in Agricultural Research, New York, 1939. McGraw-Hill Book Company, Inc.
25. Glover, J., Goodwin, T. W., and Morton, R. A.: Studies in Vitamin A. The Relationship Between Blood Vitamin A Levels and Liver Stores in Rats, Biochem. J. **41**: 97, 1947.
26. Clausen, S. W.: The Absorption of Vitamin A and Its Storage in the Tissues, The Harvey Lectures, Lancaster, Pa., 1942-43, Science Press Printing Co., p. 199.
27. Straumfjord, J. V.: Lesions of Vitamin A Deficiency. Their Local Character and Chronicity, Northwest Med. **41**: 229, 1942.
28. Straumfjord, J. V.: Vitamin A: Its Effect on Acne. A Study of One Hundred Patients, Northwest Med. **42**: 219, 1943.
29. Hickman, K. C. D., Kaley, M. W., and Harris, P. L.: Covitamin Studies. I. The Sparing Action of Natural Tocopherol Concentrates on Vitamin A, J. Biol. Chem. **152**: 303, 1944.
30. Harris, P. L., Kaley, M. W., and Hickman, K. C. D.: Covitamin Studies. II. The Sparing Action of Natural Tocopherol Concentrates on Carotene, J. Biol. Chem. **152**: 313, 1944.
31. Hove, E. L., and Harris, P. L.: Covitamin Studies. V. The Interrelation of Alpha-Tocopherol and Essential Unsaturated Fat Acids, J. Nutrition **31**: 699, 1946.
32. Chalier, J., Jeune, M., Simon, F., and Alacoque, P.: Sur les relations entre la vitamine A et le taux du cholestérol sanguin, Compt. rend. Soc. de biol. **129**: 606, 1938.
33. Wendt, H.: Beitrage zur Kenntnis des Carotin- und Vitamin A-Stoffwechsels, Klin. Wchnschr. **14**: 9, 1935.
34. Sadhu, D. P., and Brody, S.: Excess Vitamin A Ingestion, Thyroid Size and Energy Metabolism, Am. J. Physiol. **149**: 400, 1947.
35. Peters, J. R., and Van Slyke, D. D.: Quantitative Clinical Chemistry, Interpretations, vol. 1, ed. 2, Baltimore, 1946, Williams and Wilkins Company, p. 473.
36. Embree, N.: Recent Advances in the Chemistry and Nutrition of the Fat-Soluble Vitamins, Oil & Soap **23**: 305, 1946.
37. Caldwell, M. J., and Parrish, D. B.: The Effect of Light on the Stability of the Carr-Price Color in the Determination of Vitamin A, J. Biol. Chem. **158**: 181, 1945.

A COMPARATIVE STUDY OF MICRO- AND MACROELECTROPHORETIC ANALYSIS OF HUMAN AND RAT SERUM

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A DETAILED comparative study of electrophoretic analysis of protein solutions using a microcell (2 ml. capacity) and the standard macrocell (11 ml. capacity) has not been reported previously. Ross, Moore, and Miller[1] published a study on human seminal plasma in which a microcell was used. They stated that the mobilities and character of the patterns obtained agreed exactly with those observed with the 11 ml. cell. Comparative data, however, were not reported.

Since the length of the channel of the microcell is only five-ninths that of the macrocell, the time of electrophoresis becomes a limiting factor when proteins with relatively high mobilities, such as albumin, are being studied. Thus, in the case of serum, complete resolution of the components will be dependent in part upon the migration distance of the albumin component in the microcell. Aside from this, there is no theoretic basis for anticipating differences in electrophoretic analysis with the two cells. However, the fact that the two cells are different in design and manipulation would make a detailed comparative study desirable. The great value of having available a microcell for electrophoresis of small samples is obvious.

METHODS AND PROCEDURES

Use of the Microcell.—

The microcell used in this study is a commercially available model* and has the following dimensions: capacity, 2 ml.; length of center section, 5 cm.; cross sectional area, 0.30 centimeter.[2] The cell and the microelectrode vessels are mounted in a special rack adapted for the micro assembly. The center section of the microcell is moved by a pair of sliding racks and pinions, as with the macroassembly.

Since the commercially available microcells are open on both sides,† a few modifications of the usual technique are necessary. In the first place, it is necessary to lower the water level of the bath to a point just below the top of the microcell. Since with the usual low-temperature bath arrangement this will leave a portion of the cooling coil exposed, it is necessary to lower the coil to a point where it is completely immersed at the lower water level. It is obvious that unless this is done the coil will ice and freeze the stirrer. It is desirable when lowering the coil to arrange for a pair of oppositely pitched paddles on the stirrer to aid in circulating water at the top and bottom of the coil. Special masks with slits adapted to the microcell dimensions must be used. The mask over the schlieren lens and the slit mask used in scanning are modelled after the macromasks. It was found convenient to solder a pair of piano wires across the inner surface of the slit mask toward the top and bottom, to serve as reference lines.

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*Obtainable from either the Klett Manufacturing Co., New York, N. Y., or the Pyrocell Manufacturing Co., New York, N. Y.

†It is possible to close off one side of the microcell by the use of a close-fitting lucite plug inserted into the upper sections. In this way the microcell can be used in much the same manner as the macrocell. If this is attempted, the electrode vessel on the closed side must, of course, be fitted with a three-way stopcock, etc.

The microcell is set up in essentially the same manner as is the larger cell.[2] The electrode vessels are attached and filled with particular care to remove entrapped air bubbles from the small side arms. The cell carrier is placed in the thermostat; saturated salt solution is added to surround the electrodes; and the whole system is allowed to equilibrate. Before aligning the cell, it has been found necessary to remove the excess buffer from the bridge between the two channels of the top section. This is most readily effected by blotting with a piece of filter paper. Because of differences in the specific gravity of the protein and the buffer solutions on the two sides of the cell, it is advisable to equalize the hydrostatic pressure on the two sides by removing a small amount of buffer from the top section of the protein side before aligning the cell. This serves to prevent a rapid shift in the position of the boundaries once they are formed. As a convenient method for bringing the boundaries into view, one end of a piece of fine glass capillary is attached by means of rubber tubing to the compensating syringe and the drawn-out end is inserted directly into the top section of the cell on the ascending (anode) side. The compensator gears are then reversed, whereupon a slow and regular withdrawal of fluid takes place.

Once the boundaries are in view, the starting position and base line pictures can be taken. Electrophoresis is then carried out in the usual manner except that the voltage is reduced so as to maintain approximately the same potential gradient of 5 to 6 volts per centimeter. Because of the smaller cross sectional area of the microcell, a current of 8 Ma. was maintained throughout the run, rather than the 15 Ma. used with the macrocell. Under these conditions the electrophoresis time for serum is limited to about ninety minutes because of the short length of the center channel of the microcell.

In all instances serum samples were diluted with two parts of buffer before dialysis.

RESULTS

In Table I are listed values obtained with ten different pathologic human sera using both the micro- and macrocell. As can be seen, the correlation co-

TABLE I. COMPARISON OF MACRO- AND MICROELECTROPHORETIC ANALYSIS OF PATHOLOGIC HUMAN SERUM

	PER CENT COMPOSITION									
	ALBUMIN		ALPHA–1		ALPHA–2		BETA		GAMMA	
NO.	MACRO	MICRO	MACRO	MICRO	MACRO	MICRO	MACRO	MICRO	MACRO	MICRO
1	29.4	31.4	14.4	12.6	15.2	12.9	15.6	23.8	25.4	19.3
2	26.4	30.2	8.9	8.5	16.4	17.7	24.6	23.8	23.7	19.8
3	46.1	49.0	12.5	10.5	16.2	14.3	14.5	15.9	10.7	10.3
4	31.4	30.4	15.9	17.5	19.2	17.5	18.0	22.9	15.5	11.8
5	40.3	45.3	9.7	7.9	19.7	19.6	17.9	16.8	12.4	10.4
6	44.4	46.8	13.1	13.2	12.3	12.4	17.4	17.7	12.8	9.9
7	51.4	49.9	9.8	9.9	10.0	10.2	16.1	17.8	12.7	12.2
8	23.3	24.8	11.3	11.5	13.4	10.9	20.1	22.6	31.9	30.2
9	42.7	42.7	8.7	8.6	12.4	12.7	19.9	20.5	16.3	15.5
10	52.4	51.4	6.8	6.2	9.8	9.1	20.6	23.3	10.4	10.0
Mean	38.8	40.2	11.0	10.6	14.5	13.7	18.5	20.5	17.2	14.9
S.D.	9.8	9.4	2.7	3.1	3.30	3.33	2.79	2.94	6.9	6.2
r	+0.987		+0.850		+0.920		+0.575		+0.975	
p	<.01		<.01		<.01		<.05		<.01	

Diagnosis of cases as follows: 1, nonlipoid histiocytosis (Letterer-Siwe's disease); 2, carcinoma of esophagus with metastases; 3, agranulocytosis with terminal lobar pneumonia; 4, angiomatous mesothelioma of pelvis; 5, localized Hodgkin's disease with infiltration of lungs; 6, arteriosclerosis, generalized; 7, myelogenous leucemia; 8, hypernephroma with metastases; 9, hypertensive and arteriosclerotic heart disease with multiple pulmonary emboli; 10, bronchopneumonia. Electrophoretic analyses of cases 1 to 9 have been previously reported.[3]

$$r = \text{Correlation coefficient} = \frac{\Sigma x_1 y_1}{N \sigma x \sigma y} \quad \text{where} \quad \begin{matrix} x_1 = \bar{x} - x \\ y_1 = \bar{y} - y \end{matrix}$$

$$\sigma x = \text{Standard deviation (S.D.) } x = \sqrt{\frac{\Sigma x^2}{N}}$$

p = Probability of chance variation, obtained by use of r values and a table of probabilities. p Value of <.05 indicates the probability of chance variation of less than 5 in 100; p value of <.01 indicates chance variation of less than 1 in 100.

efficients are high in all instances except in the case of the beta globulin values. The known difficulty of accurately defining and measuring the beta globulin area on the descending side in all probability accounts for the less satisfactory but still significant correlation of these values.

In Table II are listed two sets of values taken from the same individual. The macrodeterminations represent three different samples taken within a period of one month; the microdeterminations represent triplicate determinations on a single sample taken from the same individual one year later. It is apparent that the range of variation within the two sets of determinations is of the same order in both the macro- and the microruns.

TABLE II. COMPARISON OF MACRO- AND MICROELECTROPHORETIC ANALYSIS OF NORMAL HUMAN SERUM

NO.	PER CENT COMPOSITION				
	ALBUMIN	ALPHA–1	ALPHA–2	BETA	GAMMA
			Macro		
1	61.2	5.0	8.1	13.8	11.9
2	60.2	6.2	8.2	12.2	13.2
3	61.0	5.6	7.6	12.4	13.4
Average	60.8	5.6	8.0	12.8	12.8
			Micro		
4	65.7	4.0	7.0	12.5	10.8
5	63.8	4.6	7.7	12.0	11.9
6	63.6	5.0	7.8	11.3	12.3
Average	64.4	4.5	7.5	11.9	11.7

In Table III the per cent composition of normal rat serum using the micro- and macrocell is shown. The mean values and standard deviations of the two sets of data are of the same order of magnitude. While these data do not represent duplicate determinations, one micro and the other macro, on the same

TABLE III. COMPARISON OF MACRO- AND MICROELECTROPHORETIC ANALYSIS OF NORMAL RAT SERUM

NO.	PER CENT COMPOSITION				
	ALBUMIN	ALPHA–1	ALPHA–2	BETA	GAMMA
			Micro		
1	50.0	18.0	13.8	12.1	6.1
2	51.4	18.0	10.4	16.2	4.0
3	49.8	16.4	11.1	17.6	5.1
4	52.8	17.7	8.9	17.0	3.6
5	50.4	20.7	9.4	13.6	5.9
6	49.3	21.1	10.2	15.5	3.8
Mean	50.6	18.6	10.6	15.4	4.8
S.D.	1.05	1.68	1.45	1.92	1.00
			Macro		
1	48.4	19.1	13.0	14.0	5.5
2	46.9	20.5	10.8	17.3	4.5
3	53.4	16.5	9.7	15.2	5.2
4	50.5	16.7	11.0	15.9	5.9
5	50.6	16.4	10.4	18.4	4.2
6	49.4	17.7	7.2	18.3	7.4
7	51.7	15.9	7.3	17.5	7.6
Mean	50.1	17.5	9.9	16.6	5.7
S.D.	1.54	1.56	1.93	1.54	1.22

serum, they indicate that in a series of determinations using normal rat serum the percentage compositions will be comparable whether determined with the micro- or the macrocell.

A comparison of mobility values obtained with the micro- and macrocell with rat and human serum is shown in Table IV. While the mean values are in reasonably close agreement, it is of interest that the standard deviations in the case of the rat serum macrodeterminations are considerably higher than those for the microdeterminations. In all probability a larger series of samples would not show this.

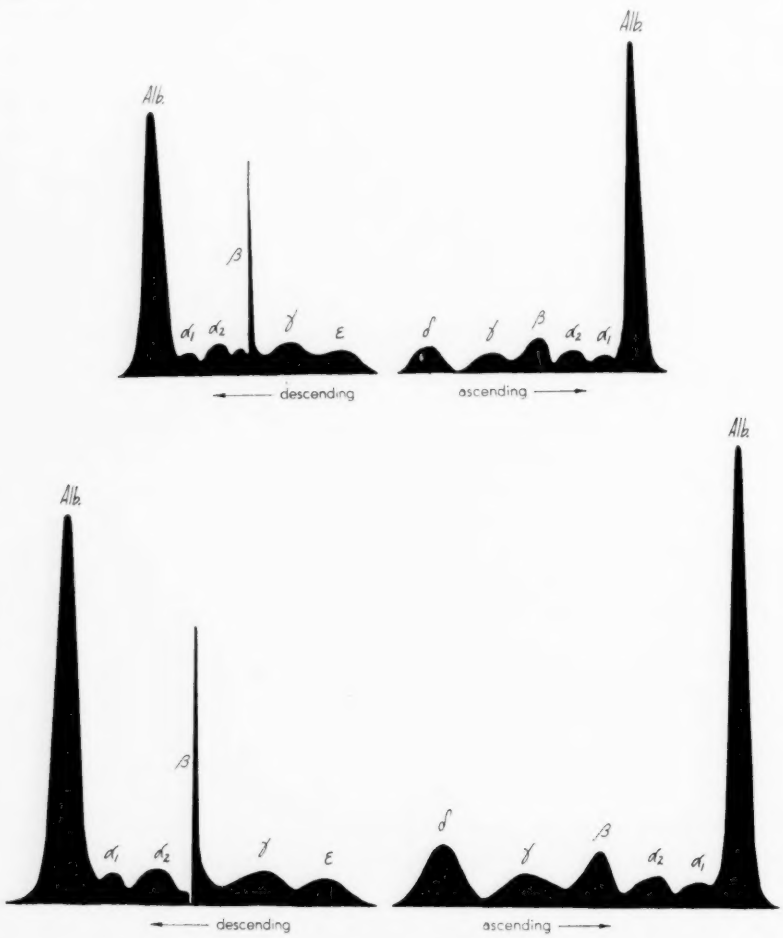

Fig. 1.—Comparison of macro- and microelectrophoretic patterns of normal human serum. Electrophoresis time: macro, 150 minutes; micro, 90 minutes. Protein concentration 2 Gm. per 100 milliliters. Relative dimensions of patterns are maintained.

In Fig. 1 tracings of a macro- and a microelectrophoretic pattern of normal human serum from the same individual are reproduced. It is apparent that resolution after 90 minutes with the microcell compares favorably with that of the macrocell, run for 150 minutes.

TABLE IV. COMPARISON OF MOBILITY VALUES OBTAINED WITH MACRO- AND MICROCELLS

	MOBILITIES × 10^5 (CM.^{2} PER VOLT SECOND)									
	ALBUMIN		ALPHA–1		ALPHA–2		BETA		GAMMA	
	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.
Human										
micro	6.7	0.35	5.6	0.27	4.7	0.22	3.2	0.25	1.3	0.26
macro	6.7	0.33	5.7	0.32	4.5	0.30	3.2	0.30	1.4	0.32
Rat										
micro	6.1	0.17	5.4	0.12	4.5	0.20	2.9	0.11	1.7	0.15
macro	6.1	0.32	5.4	0.37	4.3	0.35	2.9	0.25	1.7	0.31

In Fig. 2 tracings of a macro- and a microelectrophoretic pattern of two different normal rat sera are shown. It is to be noted that while rat serum does not show the ready resolution seen with human serum, the micro- and macro-patterns are quite comparable.

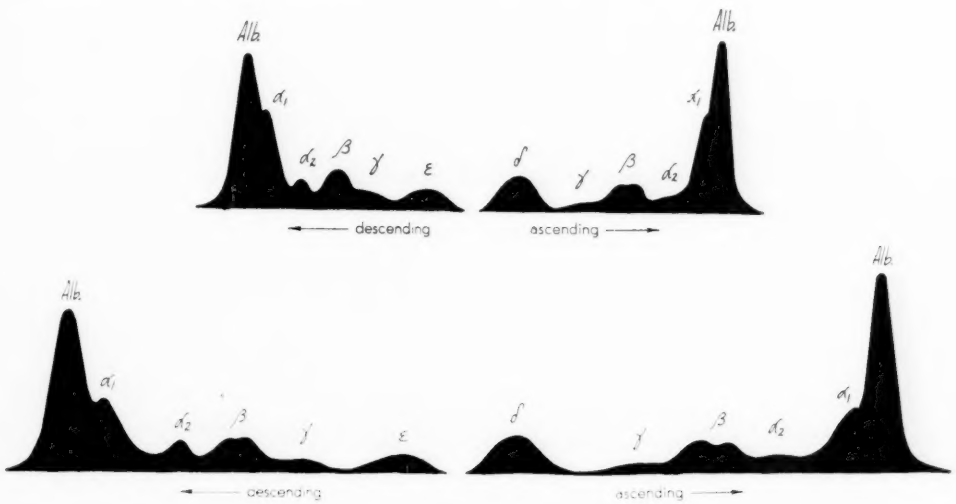

Fig. 2.—Comparison of macro- and microelectrophoretic patterns of normal rat serum. Electrophoresis time; macro, 180 minutes; micro, 90 minutes. Protein concentration, 2 Gm. per 100 milliliters. Relative dimensions of patterns are maintained.

DISCUSSION

The comparative data for micro- and macroelectrophoretic analyses of serum proteins reported here indicate that the values obtained by the two methods are in close agreement. In instances in which one is dealing with samples of serum of small volume the practical importance of the microcell is apparent. However, it is important to emphasize that a series of comparative determinations of the type reported here may be required in any study in which the two cells are to be used, particularly when quantitative differences of small magnitude are anticipated. It should also be pointed out that protein mixtures other than serum have not been extensively studied and, therefore, preliminary investigation of completeness of resolution of the component proteins must be established beforehand.

SUMMARY

1. Electrophoretic patterns of normal and pathologic human serum and normal rat serum have been compared using a micro- and a macroelectrophoresis cell. The mobilities and per cent composition of the components are in close agreement by the two methods.

2. Some technical aspects of the use of the microcell are presented and discussed.

REFERENCES

1. Ross, V., Moore, D. H., and Miller, E. G., Jr.: Proteins of Human Seminal Plasma, J. Biol. Chem. **144**: 667, 1942.
2. Longsworth, L. G.: Recent Advances in the Study of Proteins by Electrophoresis, Chem. Rev. **30**: 323, 1942.
3. Cohen, P. P., Thompson, F. L., and Nitshe, G. A., Jr.: Electrophoretic Analysis of Ante- and Postmortem Serum in Different Diseases, J. Clin. Investigation **26**: 820, 1947.

STERNAL MARROW HEMOSIDERIN

A Method for the Determination of Available Iron Stores in Man

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A KNOWLEDGE of the available iron stores is useful in the management of anemia. The extreme manifestations of iron lack such as koilonychia, glossitis, dysphagia, and microcytic hypochromic red cells are the result of a long existing deficiency. Iron excess is even more difficult to recognize than its lack, except in cases of hemochromatosis or severe hemosiderosis where there are pigmented deposits in the skin or in the urinary sediment. The majority of clinical problems in iron metabolism lie between these extremes, and in this group the examination of sternal marrow has proved to be a convenient and reliable index of iron deficiency or iron excess.

MATERIAL AND METHODS

One or more sternal punctures were performed on sixty-three individuals. This group included eleven normal subjects, sixteen patients with iron deficiency anemia, eleven with pernicious anemia, six with uremia, five with subacute or chronic infection, five with cirrhosis of the liver, three with lupus erythematosus, two with hemochromatosis, and four with hemosiderosis secondary to multiple transfusions. Only clear-cut examples were selected for this report, in which there was a careful history relevant to blood loss and iron intake by mouth or parenterally. Patients with iron deficiency anemia showed typical microcytosis and hypochromia which were associated with a low serum iron in those patients in whom the serum iron was determined. The diagnosis of pernicious anemia was confirmed by the characteristic cell indices and blood cytologic changes, megaloblastic sternal marrow, and a satisfactory reticulocyte response to intramuscular liver. Those with uremia presented the clinical picture of severe renal damage with a blood urea nitrogen in the neighborhood of 100 mg. per cent. Patients in the infectious group had a daily oral temperature of 100°F. and above for more than two weeks. All the patients with cirrhosis had obvious impairment of function tests and other stigmas of chronic liver disease. Both patients with hemochromatosis presented the typical hepatic lesions of that disease by biopsy and had a high serum iron with complete saturation of the iron binding protein characteristic of hemochromatosis.[1] The four patients with extensive hemosiderosis had had twenty-eight, eleven, fifty, and forty-nine transfusions, respectively.

Sternal punctures were performed.‡ After preliminary novocain infiltration, a 2½ cm. No. 14 needle with a short bevel is introduced into the sternal marrow cavity in the midline at the level of the second interspace. The stylet is then withdrawn and 4 c.c. of marrow and blood are drawn into a 20 c.c. syringe containing 6 c.c. of 4 per cent sodium citrate. This mixture is then ejected into a large watch glass and cover slip films are made from marrow fragments picked up with a capillary pipette.

These preparations are examined microscopically both unstained and after staining with hydrochloric acid and ferrocyanide (Berlin blue stain).§ In the unstained preparations the hemosiderin appears as golden-yellow granules under reduced illumination. In the stained preparation the granules take a blue stain. Not infrequently structures other

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‡According to the technique employed by Dr. Joseph Ross.

§The stain was prepared as follows: 4 Gm. of potassium ferrocyanide were diluted to 20 c.c. with water. Concentrated hydrochloric acid was added until a white precipitate formed. This was filtered and the smears covered with the filtrate for thirty minutes.

than hemosiderin will stain, usually in proportion to the amount of iron present. These artifacts can be recognized with experience and by comparing the two types of preparation. In iron deficiency, pale-yellow granules which do not take iron stain are sometimes seen. These may be analogous to the protein granules of hemosiderin remaining after removal of iron by extraction with 10 per cent hydrochloric acid.[2] The pale-yellow color is probably due to adsorbed bilirubin. The smears were graded according to the amount of iron present:

0	None	4	Moderately heavy
1	Very slight	5	Heavy
2	Slight	6	Very heavy
3	Moderate		

RESULTS

In Fig. 1, examples of these marrow preparations are demonstrated. Each marrow was graded independently by each of us and there was close agreement in most of the material examined. The independent ratings rarely varied more than one grade in either direction.

In the normal group the marrow iron was graded as 1 or 2 in every case except one which received a grade of 3 (Table I). All of the patients with

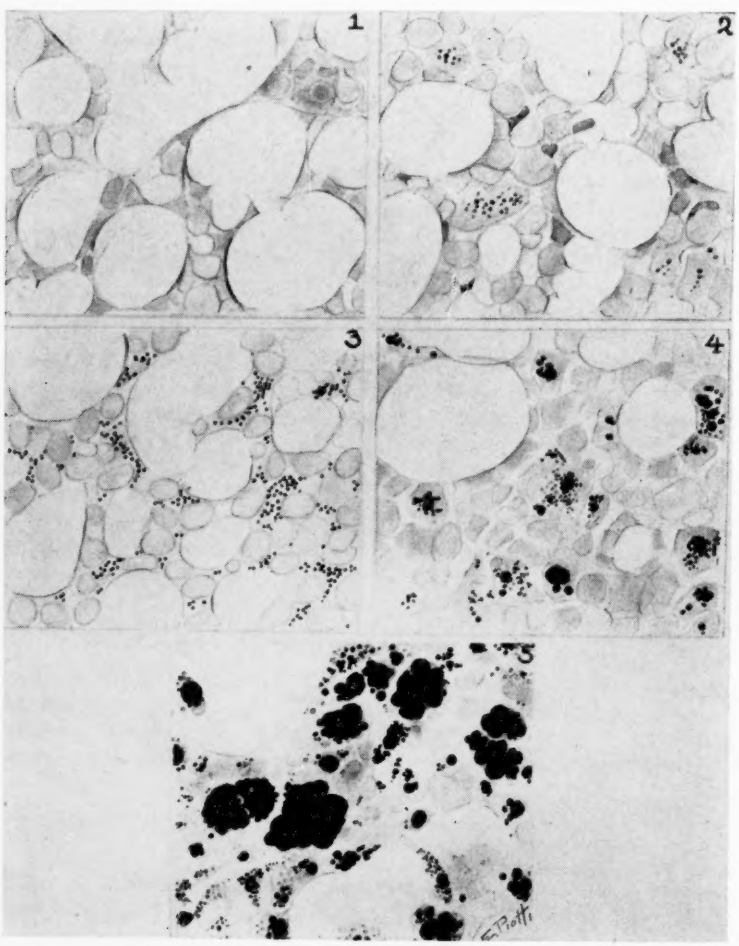

Fig. 1.—Sternal marrow preparations in which the hemosiderin deposits are illustrated as black granules. *1.* Grade 0, patient with severe iron deficiency. *2.* Grade 2, normal subject. *3.* Grade 3, patient with pernicious anemia. *4.* Grade 4, patient with chronic infection. *5.* Grade 6, patient with multiple transfusions.

TABLE I. NORMAL SUBJECTS

SUBJECT	SEX	AGE	HB. (GM.)	MCV* (CU. μ)	MCH† $\gamma\gamma$	MCHC‡ (%)	DIAGNOSIS	STERNAL MARROW IRON
W. M.	M	25	15.6	89	31	35	Normal	2
C. O.	M	24	15.8	88	30	33	Normal	1
C. F.	M	32	16.0	82	28	34	Normal	1
A. M.	M	29	17.5	91	32	39	Normal	2
P. McD.	M	21	13.8				Acute pharyngitis	2
G. T.	M	48	19.2				Obesity	3
J. F.	M	30	15.0				Cholelithiasis	2
F. W.	F	44	14.7				Dermatitis	2
E. H.	F	56	15				Parkinsonism	1
J. M.	M	61	16.9				Hypertensive cardiovascular disease	2
M. S.	M	62	16.4				Generalized arteriosclerosis, cerebral arteriosclerosis	2

*Mean corpuscular volume.
†Mean corpuscular hemoglobin.
‡Mean corpuscular hemoglobin concentration.

TABLE II. IRON DEFICIENCY ANEMIA

SUBJECT	AGE	SEX	HB. (GM.)	MCV* (CU. μ)	MCH† ($\gamma\gamma$)	MCHC‡ (%)	SOURCE OF BLOOD LOSS	STERNAL MARROW IRON
J. C.	59	F	5	54	14	25	?	0
R. C.	28	F	12.5				Thrombocytopenic purpura with menorrhagia	0
T. B.	68	F	7.9	68	19	28	Carcinoma of colon	0-1§
J. D.	47	M	7.2	63	16	26	?	0
M. H.	77	F	6.8	63	17	26	Achlorhydria	0
N. F.	14	F	9.6	75	23	31	?	0-1§
V. C.	18	F	13.4	71	22	31.5	?	0-1§
A. Y.	33	F	5.5	65	18	27	?	0-1§
J. M.	46	M	8.5	98	29	30	Acute bleeding ulcer	0
R. N.	36	M	8.2	88	30	35	Acute bleeding ulcer	0-1§
M. M.	35	F	9.0				Idiopathic thrombocytopenia	0
E. L.	36	M	10.1	82	23	29	Gastrointestinal bleeding	1§
M. B.	13	F	10.6				Idiopathic thrombocytopenic purpura	0-1§
J. S.	73	M	9.7				Hiatus hernia	0
E. McL.	62	F	7.8	71	18	25	?	0
A. B.	37	F	5.5	60	14	24	?	0

*Mean corpuscular volume.
†Mean corpuscular hemoglobin.
‡Mean corpuscular hemoglobin concentration.
§Those with sternal marrow iron grade 1 showed pale-yellow granules which stained little or not at all with the iron stain.

TABLE III. PERNICIOUS ANEMIA

SUBJECT	AGE	SEX	HB. (GM.)	MCV* (CU.μ)	MCH† ($\gamma\gamma$)	MCHC‡ (%)	STERNAL MARROW IRON
P. A.	58	F	10.2	112	37	34	3
M. McM.	71	M	5.8	126	43	34	4
K. MacL.	54	M	11.2	106	37	35	4+
M. G.	51	F	7.9				3+
H. D.	32	F	6.4	100	36	36	3
M. L.	73	M	5.1	115	40	36	5
M. P.	69	F	8.0	99	38	38	5
H. P.	68	M	8.5	135	45	34	3
V. V.	58	F	9.1	100	36	36	3+
C. C.	56		8.0	110	36	33	4+
J. R.	76	F	12.3	118	42	35	0-1

*Mean corpuscular volume.
†Mean corpuscular hemoglobin.
‡Mean corpuscular hemoglobin concentration.

TABLE IV. UREMIA

SUBJECT	AGE	SEX	HB. (GM.)	MCV* (CU.μ)	MCH† (γγ)	MCHC‡ (%)	DEGREE OF UREMIA	STERNAL MARROW IRON
B. R.	46	F	7.6	90	27	31	BUN‖ 113§	0-1
B. A.	67	F	4.7				BUN 110	0-1
Z. A.	56	F	11.0	72	26	36	BUN 50	0
B. B.	30	M	11.0				BUN 103	4+
A. M.	79	M	8.5				BUN 90	3
G. G.	49	M	6.5	82	26	32	BUN 190	2+

*Mean corpuscular volume.
†Mean corpuscular hemoglobin.
‡Mean corpuscular hemoglobin concentration.
§Recurrent epistaxis.
‖Blood urea nitrogen.

TABLE V. CIRRHOSIS

SUBJECT	AGE	SEX	HB. (GM.)	MCV* (CU.μ)	MCH† (γγ)	MCHC‡	BLOOD LOSS	STERNAL MARROW IRON
H. F.	64	M	10.5	109	34	31	None known	4
G. McG.	62	F	7.4	109	36	32	None known	4
A. W.	51	F	7.5	102	29	30	Guaiac-positive stools	0-1
							None known	3
D. M.	64	M	12.0					
J. W.	69	M	10.7	124	41	33	None known	1-2

*Mean corpuscular volume.
†Mean corpuscular hemoglobin.
‡Mean corpuscular hemoglobin concentration.

TABLE VI. INFECTION

SUBJECT	AGE	SEX	HB. (GM.)	MCV* (CUμ)	MCH† (γγ)	MCHC‡ (%)	DIAGNOSIS	STERNAL MARROW IRON
N. D'Q.	25	F	9.7	85	29	36	SBE§ six months	3-4+
J. I.	60	F	9.4	97	33	32	Chronic pulmonary disease, five years	4+
C. J.	65	M	11.0	92	29	31	Severe pneumococcal pneumonia	5
S. D.	42	M	13	87	28	33	SBE three months	3+
H. S.	54	F	9.5	79	25	32	PUO‖ two months	3+

*Mean corpuscular volume.
†Mean corpuscular hemoglobin.
‡Mean corpuscular hemoglobin concentration.
§Bacterial endocarditis.
‖Fever.

TABLE VII. DISSEMINATED LUPUS ERYTHEMATOSUS

SUBJECT	AGE	SEX	HB. (GM.)	MCV* (CUμ)	MCH† (γγ)	MCHC‡ (%)	STERNAL MARROW IRON
N. McG.	39	F	8.3	92	27	30	4+-5-
D. S.	39	F	5.9	92	29	32	5
E. R.	19	F	9.7				5

*Mean corpuscular volume.
†Mean corpuscular hemoglobin.
‡Mean corpuscular hemoglobin concentration.

iron deficiency anemia showed a marrow iron of 0 or 1 (Table II). Of the eleven patients with pernicious anemia, only one showed a marrow iron of less than 3, and five of the eleven showed a marrow iron of 4 or more (Table III). In the patients studied, the granules presented a somewhat characteristic appearance in that they were small, numerous, and of uniform size (Fig. 1, *3*). In the patients with uremia the sternal marrow iron ranged from 0 to 4 plus (Table IV). The cirrhotic patients showed a normal or increased iron in three of five patients (Table V). Five patients with infection showed an increase in the marrow iron (Table VI). Three patients with lupus erythematosus showed a heavy marrow iron deposition (Table VII). The heaviest iron deposition, grades 5 and 6, was seen in the patients with hemochromatosis and hemosiderosis (Tables VIII and IX).

TABLE VIII. HEMOCHROMATOSIS

SUBJECT	AGE	SEX	HB. (GM.)	MCV* (CU.μ)	MCH† ($\gamma\gamma$)	MCHC‡ (%)	STERNAL MARROW IRON
E. M.	58	M	15.2	96	31	33	5+
M. B.	62	M	14.8	94	33	35	5+

*Mean corpuscular volume.
†Mean corpuscular hemoglobin.
‡Mean corpuscular hemoglobin concentration.

TABLE IX. HEMOSIDEROSIS

SUBJECT	AGE	SEX	HB. (GM.)	MCV* (CU.μ)	MCH† ($\gamma\gamma$)	MCHC‡ (%)	NUMBER OF TRANSFUSIONS	STERNAL MARROW IRON
A. A.	56	M	5.5	93	27	29	28	5
H. K.	66	M	7.6	115	38	33	11	5
J. S.	55	M	6.4	100	34	34	50	5
P. M.	23	F	5	101	32	32	3 (at onset of anemia)	2+
	25	F	10.0	91	29	32	49 (18 months later)	6+

*Mean corpuscular volume.
†Mean corpuscular hemoglobin.
‡Mean corpuscular hemoglobin concentration.

COMMENT

Storage iron in man exists as ferritin and hemosiderin. The precise relationship between them is not clear. It would appear, however, that ferritin represents a labile or active form of iron storage and that hemosiderin is a less active form of iron storage, present only when there are adequate ferritin stores.[3] The studies of Bogniard and Whipple have demonstrated that hemosiderin is mobilized for hemoglobin production when needed by the body.[4] Of these two forms of iron storage, hemosiderin is the only one morphologically identifiable. These yellow or brownish-yellow granules are found throughout the reticuloendothelial system and macrophages of the body. The sternal marrow provides a readily accessible portion of the reticuloendothelial system for biopsy.

In this group of patients, the sternal iron has been consistent with the alteration of iron storage characteristic of these diseases. In infection there was

an increase in hemosiderin above the amount normally seen. The progressive hemosiderosis in this condition appears to be due to an increased affinity of the tissues for iron.[5] In pernicious anemia there is likewise an increase in tissue iron due in part at least to storage of red cell iron in the tissues. In hemochromatosis, sternal iron is increased as are other iron stores throughout the body. Multiple transfusions provide even heavier deposits of iron since the iron provided by the donated red cells is not excreted in appreciable amounts from the body.[7] Patients with cirrhosis and nephritis have variable iron stores due to the variable bleeding in these diseases. Iron deficiency, in contrast to the normal controls and the other diseases described, shows an absence of iron staining granules. In this group of patients subsequent iron therapy was effective in alleviating the anemia.

It is to be expected that the balance of blood loss and iron absorption over a period of years will vary considerably from person to person, although this is remarkably well regulated by the absorptive mechanism.[6] However, certain conditions will modify the stores to an extent which overshadows these normal variations. That a clear-cut differentiation of the anemia of infection from that of iron deficiency usually may be made is evident from comparing Table II and VI. Hemochromatosis also may be excluded in patients with cirrhosis if the marrow iron is not increased (Tables V and VIII).

A number of patients referred to us with mild anemias reputed to be iron refractory were shown by marrow puncture to have adequate or increased iron stores. Those anemias were felt to be due to other causes, that is, obscure infection or damaged bone marrow. In our experience, only patients with absent marrow iron will be benefited by iron therapy.

SUMMARY

A method of estimating tissue iron stores by sternal puncture has been described.

A group of sixty-three patients have been studied and the hemosiderin content of the marrow was found to parallel the anticipated iron storage in these diseases.

The presence or absence of iron in the marrow may be regarded as an index of the need for iron therapy in anemia.

REFERENCES

1. Finch, C. A., and Rath, C. E., Jr.: To be published.
2. Cook, S. F.: The Structure and Composition of Hemosiderin, J. Biol. Chem. **82**: 595-609, 1929.
3. Granick, S.: Ferritin: Its Properties and Significance for Iron Metabolism, Chem. Rev. **38**: 379-403, 1946.
4. Bogniard, R. P., and Whipple, G. H.: Iron Content of Blood Free Tissues and Viscera; Variations Due to Diet, Anemia and Hemoglobin Injections, J. Exper. Med. **55**: 653-665, 1932.
5. Greenberg, G. R., Ashenbrucker, H., Lauritsen, M., Worth, W., Humphreys, S. R., and Wintrobe, M. M.: The Anemia of Infection. V. Fate of Injected Radioactive Iron in the Presence of Inflammation, J. Clin. Investigation **26**: 121-125, 1947.
6. Hahn, P. F., Bale, W. F., Ross, J. F., Balfour, W. M., and Whipple, G. H.: Radioactive Iron Absorption by Gastro-Intestinal Tract; Influence of Anemia, Anoxia, and Antecedent Feeding Distribution in Growing Dogs, J. Exper. Med. **78**: 169-188, 1943.
7. McCance, R. A., and Widdowson, E. M.: Iron Excretion and Metabolism in Man, Nature **152**: 326-327, 1943.

OCCURRENCE OF TOXOPLASMA NEUTRALIZING ANTIBODIES IN VARIOUS DISEASE CONDITIONS

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THE toxoplasma neutralization test has been used extensively as an aid in the diagnosis and recognition of nonfatal and inapparent infection in man. With this procedure or the unequivocal demonstration of parasites in tissues either by animal inoculation or by histologic methods, it has been found that toxoplasmic infection can be responsible for many conditions such as: (1) congenital encephalomyelitis, which becomes manifest either in utero or shortly after birth; (2) acute encephalitis in children; (3) infection in adults resembling Rocky Mountain spotted fever; and (4) mild or inapparent infection. An investigation of the occurrence of toxoplasma neutralizing antibodies in the blood of certain selected individuals is the basis of this report.

The method for performing the neutralization test has been reported in detail by Sabin[1] and consists of the following procedure with minor modifications. A freshly prepared 10 per cent toxoplasma-infected mouse brain suspension in saline is allowed to sediment spontaneously for one-half hour. The supernatant fluid is drawn off and further dilutions of 1:50, 1:500, and 1:5,000 are made. Equal volumes of these dilutions (0.15 c.c.) are added to undiluted serum (or Tyrode's solution for the controls), giving final dilutions of 1:20, 1:100, 1:1,000, and 1:10,000. The mixtures are incubated at room temperature for approximately one-half hour at which time 0.2 c.c. amounts are injected intracutaneously on the back of a rabbit. The lesions which develop are measured at the end of seven days. The inhibition of skin lesions by the test sera are compared with control lesions and the degree of neutralization determined. The criteria for interpretation of results are those advanced by Sabin.[1]

Serum specimens were kept as fresh as possible and either used immediately or stored in dry ice until needed. They were obtained from individuals who, along with other signs, presented one or more of the following manifestations: hydrocephalus or microcephaly, cerebral calcification, chorioretinitis or other eye changes, and involvement of the central nervous system. Members of the immediate families were also examined for the presence of antibodies wherever indicated. Tests were also performed on patients with diseases of unknown etiology in order to determine a possible relationship to toxoplasmosis. Individuals suspected of having a congenitally acquired illness were also tested.

The results of the ninety tests performed with the sera from seventy-two individuals are listed in the following tables. Reported in Table I are positive results on seven patients, one month to five years of age, who presented evidence

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of having acquired the infection in utero, since they were either sick at birth or became sick shortly after birth. Six of the patients in this group had cerebral calcification; five had chorioretinitis or other signs of eye involvement; three had hydrocephalus; one had microcephaly; and six manifested convulsions or other signs of central nervous system involvement. Additional findings were a rash in two patients and splenomegaly in one patient. It is now apparent that cerebral calcification and central chorioretinitis are the important signs of congenital toxoplasmosis. One or the other or both were present in six of the patients. The seventh showed only microcephaly and mental retardation. In each instance tests of the mothers' sera showed toxoplasma neutralizing antibodies, further proof of the congenital inception of the disease. The father and an older sibling of one of the patients also developed antibodies. It is noteworthy that the serum from patient Bla.,[4] which was positive at the age of one year, still showed the presence of antibodies when tested more than five years

TABLE I. PATIENTS WHOSE SERA NEUTRALIZED TOXOPLASMA

INCEPTION	PATIENT	AGE	CHIEF FINDINGS	RESULTS OF NEUTRALIZATION TESTS WITH SERA FROM IMMEDIATE FAMILY*
Congenital	Gri.†	1 mo.	Cerebral calcification, hydrocephalus, chorioretinitis, convulsions, rash	[M] [F] [S] ⓢ
	Cle.	1 mo.	Cerebral calcification, convulsions, head rolling, apnea, xanthochromia	[M]
	Scu.	5 mo.	Microcephaly, mental retardation	[M]
	Gro.	6 mo.	Cerebral calcification, rash, splenomegaly, retrolental fibroplasia	[M]
	Kam.	2 yr.	Cerebral calcification, hydrocephalus, chorioretinitis, eyes of different size	[M] ⓕ ⓢ
	Kuh.‡	4 yr.	Cerebral calcification, chorioretinitis, convulsions, mental retardation	[M]
	Bla.§	5 yr.	Proved case; 5 years later: cerebral calcification, hydrocephalus, chorioretinitis	[M]
Probably congenital	Kre.	7 yr.	Chorioretinitis, psychomotor disturbances	--
Acquired	Lyk.	9 yr.	Encephalitis last six weeks, questionable brain tumor	ⓜ
	Jaq.	32 yr.	Mental disturbance (psychoneurotic)	--
	Par.	62 yr.	Encephalitis and mental disturbance for one week	--

*[ ], Neutralized; ○, did not neutralize; M, mother; F, father; S, sibling; —, not available.

†Proved fatal case.[2]

‡Case reported by Miller.[3]

§Case reported by Sabin.[4]

later. Inoculation of spinal fluid and blood from two of the patients (Kam. and Lyk.) into mice was without effect. However, toxoplasma were readily recovered from animals inoculated with ground suspensions of tissues obtained from patient Gri. at autopsy. Positive tests on the sera of four other patients (7 to 62 years of age) are also reported in Table I. In these patients the onset of the illness could not be determined with certainty. Patient Kre., 7 years of age, showed unilateral chorioretinitis (type not determined) and psychomotor

disturbances. The mother was not available for testing. Patient Lyk., 9 years of age, whose serum was positive, had had encephalitis for the previous six weeks. A retest several months later was again positive, while the mother's serum was negative. Included in this series are two adults: one, a 32-year-old female psychoneurotic patient with a questionable optic atrophy, and the other, a 62-year-old male who was said to have had encephalitis of about one week's duration from which he completely recovered. Two months previously he had been bitten by a cat that later died in convulsions. One cannot assume, however, that toxoplasma were the cause of these conditions because these patients might have acquired the antibody sometime in the past as a result of inapparent infection.

TABLE II. PATIENTS WITH HYDROCEPHALUS WHOSE SERA FAILED TO NEUTRALIZE TOXOPLASMA

INCEPTION	PATIENT	AGE	ADDITIONAL FINDINGS	RESULTS OF NEUTRALIZATION TESTS WITH SERA FROM IMMEDIATE FAMILY*
Congenital	Car.†	1 mo.	Macular chorioretinitis, psychomotor disturbance	--
	Kle.	3 mo.	Difficult delivery, transitory nystagmus	Ⓜ
	Mad.	6 mo.	Birth injury, subdural hematoma, spasticity	--
	Dam.	7 mo.	Mental retardation, nystagmus, tumor mass in right eye	Ⓜ
	Aut.	1 yr.	Meningocele at birth	--
	Lem.	2½ yr.	Difficult delivery, mental retardation	--
Probably congenital	Tay.	5 mo.	Mental retardation	Ⓜ
	Mil.	18 mo.	Congenital heart disease, mental retardation	--
	Rus.	20 mo.	Cerebral atrophy, ataxia, strabismus	Ⓜ
	Lit.	3½ yr.	Onset at 1 year; chorioretinitis, mental retardation, convulsions	Ⓜ Ⓢ Ⓢ

*○, Did not neutralize; M, mother; S, sibling; —, not available.
†Patient too young to develop own antibodies.

As recorded in Table II, the sera of ten children with hydrocephalus (one month to three and one-half years of age) were completely negative for toxoplasma neutralizing antibodies. In most instances the hydrocephalus resulted from developmental defects or trauma, but in two patients only the hydrocephalus was associated with chorioretinitis. Patient Car. had chorioretinitis in the macular region of both eyes and hydrocephalus at the age of 1 month. It should be pointed out, however, that the neutralization test may be negative in a newly born infant and positive when the child is several months old.[4] Thus it is of greater importance to test the mother's blood as early as possible after the birth of the baby, and the child's blood several months later. Unfortunately in this case neither the child nor the mother was available for subsequent tests. Patient Lit., with a possible onset at one year of age, developed hydrocephalus, convulsions, and chorioretinitis whose type and distribution was not determined. In this instance the sera of the child and the mother were negative. It is worth noting that in the series of children without antibodies, the mothers' sera were also found to be negative. The instances in which the sera of both mother and

child are positive therefore assume greater significance as serologic evidence for a diagnosis of congenital toxoplasmosis.

Cerebral calcification in the absence of toxoplasma neutralizing antibodies was encountered four times (Table III). In two of the patients (Fle. and Ste.) the calcification was associated with developmental defects. However, toxoplasmosis could not be excluded with certainty in two (Bel. and Nei.), one patient in particular (Bel.) having manifestations compatible with a diagnosis of toxoplasmic infection. The sera from the mother and child were sent through the regular mail during the hot summer months without any precautions for preserving the heat-labile toxoplasma antibody. Even so, they were not completely negative and it is highly probable that antibodies might have been demonstrated in a fresh specimen. Further specimens were not obtainable. Patient Nei. also gave a history compatible with a diagnosis of toxoplasmosis, but repeated tests many months apart failed to reveal neutralizing antibodies in either the mother or the child. The absence of neutralizing antibodies in a child showing the clinical signs of congenital toxoplasmosis has been reported.[1] Both the mother and child failed to show toxoplasma neutralizing antibodies on repeated tests but regularly showed the presence of complement-fixing antibodies. Again in this group the mothers as well as the offspring lacked the neutralizing antibodies.

TABLE III. PATIENTS WITH CEREBRAL CALCIFICATION WHOSE SERA FAILED TO NEUTRALIZE TOXOPLASMA

INCEPTION	PATIENT	AGE	ADDITIONAL FINDINGS	RESULTS OF NEUTRALIZATION TESTS WITH SERA FROM IMMEDIATE FAMILY*
Congenital	Fle.	½ mo.	Spina bifida, meningocele	Ⓜ
	Ste.	16 mo.	Failure to develop	Ⓜ (Equivocal)
	Bel.†	3 yr.	Bilateral macular chorioretinitis, microcornea	Ⓜ (Equivocal)
	Nei.	4 yr.	Microcephaly, mental retardation, optic atrophy	Ⓜ

Zone of calcification: Fle., right hemisphere between parietal bone and right coronal suture; Ste., frontoparietal area near left vertex; Bel., linear calcifications in the brain; Nei., walls of ventricles.

*○, Did not neutralize; M, mother.

†Serum specimens were in the mail for several days during the summer months. Results of the neutralization test were equivocal.

Negative results were obtained with the blood of six patients, 2 months to 27 years of age, with retinal lesions but without signs of hydrocephalus or cerebral calcification (Table IV). In addition to other symptoms, two members of the group, age 2 and 5 months, respectively, developed convulsions. The 5-month-old child had central choroiditis and nystagmus as well. Serum tests of the mothers of these children were likewise negative for toxoplasma antibodies. The remaining four patients were of sufficient age to develop antibodies but, since none were found, no attempt was made to examine the mothers' blood. The type and distribution of lesions in these individuals as well as the possible

TABLE IV. PATIENTS WITH EYE CHANGES WHOSE SERA FAILED TO NEUTRALIZE TOXOPLASMA

INCEPTION	PATIENT	AGE	FINDINGS	RESULTS OF NEUTRALIZATION TESTS WITH SERA FROM IMMEDIATE FAMILY*
Congenital	Mol.	2 mo.	Eyes closed, slight retinal changes, convulsions	Ⓜ
	Ans.	5 mo.	Bilateral macular chorioretinitis, nystagmus, convulsions	Ⓜ
	Hil.	3½ yr.	Failure to develop since birth, chorioretinitis (2 years later)	--
Probably congenital	Gai.	2 yr.	Bilateral chorioretinitis (diffuse, pigmented)	--
Acquired	Glo.†	19 yr.	Active bilateral macular chorioretinitis	--
	Rom.	27 yr.	Bilateral chorioretinitis (hemorrhagic) in last year	--

*○, Did not neutralize; M, mother; —, not available.
†Equivocal on repeated tests.

onset are listed in Table IV. The chorioretinitis was first noted about two years after birth in patient Hil. In the adults, Glo. and Rom., the chorioretinitis was active and had developed within the past year.

The determination of neutralizing antibodies was extended to include disease conditions in which a definite etiology could not be established. When individuals with signs of hydrocephalus, cerebral calcification, and chorioretinitis had been eliminated, there was still a large group (ages 7 months to 74 years) that showed negative neutralization tests. In many instances, especially in children with congenitally acquired diseases, the tests were performed to exclude the possibility of toxoplasmosis. The primary diagnosis or chief findings are listed in Table V. In this group three patients had prolonged encephalitis, six had encephalopathies of obscure etiology, three were believed to have Hodgkin's disease, one was a patient with eosinophilia, and one with hepatosplenomegaly; there was also an individual who had been exposed to toxoplasma in the laboratory for many years.

TABLE V. CONDITIONS WHICH FAILED TO PRODUCE TOXOPLASMA NEUTRALIZING ANTIBODIES

PATIENT	AGE	DIAGNOSIS
Dho.	7 mo.	Familial demyelinating sclerosis
Aho.	18 mo.	Familial demyelinating sclerosis
Vos.	2 yr.	Congenital bone abnormalities, mental retardation
But.	2½ yr.	Encephalitis last 6 weeks (convulsions, ataxia, spasticity, pleocytosis)
Wal.	5 yr.	Right cerebral atrophy, epilepsy, convulsions, spasticity
Smi.	8 yr.	Eosinophilia
Tem.	8 yr.	Prolonged encephalitis, cortical atrophy, mental retardation
Bal.	14 yr.	Brain abscess
Dav.*	15 yr.	Acute encephalitis (toxoplasma isolated from animals inoculated with C. S. F.); patient tested seven years later
Fla.	18 yr.	Hodgkin's disease
Tro.	36 yr.	Hodgkin's disease
Lap.	38 yr.	Hodgkin's disease
Ruc.	37 yr.	Laboratory exposure for many years
Sak.	50 yr.	Epileptic seizures, abortions
Hes.	74 yr.	Hepatosplenomegaly, empyema of gall bladder

*Case reported by Sabin.[5]

DISCUSSION

With the aid of quantitative methods, Sabin and Olitsky[6] were able to demonstrate the development of toxoplasma neutralizing antibodies in experimentally infected monkeys. Later, utilizing the same method, Sabin[5] was able to show the presence of such antibodies in a human patient with toxoplasmosis, and this was soon corroborated by Cowen, Wolf, and Paige.[7] The latter were able to show not only that antibodies were present in the young patients but in some of the mothers as well, thus giving additional evidence for the belief that the disease was congenitally acquired by those patients. Certain discrepancies regarding the presence of neutralizing antibodies were explained when Sabin and Ruchman[8] discovered the extreme lability of the antibody and the ease with which it disappeared from stored serum unless proper precautions were taken for its preservation. Sera kept frozen in dry ice or lyophilized were found to retain their antibody content for long periods of time. With due regard for the instability of the antibody, it was noted that neutralizing substances appeared in the blood stream of monkeys within two weeks after infection and persisted for over a year, which was the longest interval tested. It was also observed that the presence of antibody was not associated with the persistence of parasites in the tissues of the monkeys. Having ascertained that the neutralization test yielded reproducible results, Sabin[1] extended the work to include sera obtained from human beings. It was found that neutralizing antibodies were almost regularly present in infants and children showing psychomotor disturbances with or without hydrocephalus or microcephaly only when these were associated with cerebral calcification or chorioretinitis or both. Corroboration was obtained of the presence of antibodies in the mothers of affected children. Occasionally other members of the immediate family were found to be positive. In a high proportion of patients examined, neutralizing antibodies were found in patients with obscure encephalopathies, in mothers who gave birth to anencephalic, microcephalic, or hydrocephalic infants, and in older children with chorioretinitis as well as some of their mothers. The latter were made the basis of a separate report by Vail and co-workers.[9] Callahan[10] studied the sera of one hundred apparently well adults and found that only 2 per cent were able to neutralize the effects of toxoplasma. Heidelman[11] corroborated the finding of a high incidence of positive sera in patients with congenital chorioretinitis as well as in many of their mothers. The incidence was approximately 10 per cent in cases of acquired chorioretinitis and much less in patients with acquired anterior uveitis. A case diagnosed clinically as toxoplasmic in origin was used by Adams and associates[12] to study the sera obtained from members of the immediate family. They were able to show the presence of antibodies in the patient, the mother, and nine of ten siblings. Finally, Johnson[13] corroborated the finding of a high incidence of positive sera among patients with active or inactive central chorioretinitis, especially when cerebral calcification was also present. Among the sera of twenty-eight patients (15 to 50 years of age) with macular chorioretinitis alone, a total of 16, or 57 per cent, was positive. The sera from four individuals (3 to 5 years of age at first examination) who had cerebral calcification in addition to the chorioretinitis were positive. Again the

blood of the mothers (two of two tested) and of some of the siblings (eight of ten tested) of such patients was positive.

The results reported in this series of toxoplasma neutralization tests corroborate the general finding of positive sera among patients with hydrocephalus or microcephaly and psychomotor disturbances when these are associated with chorioretinitis, particularly in the macular region of the eye, and with cerebral calcification. The high incidence of positive antibody tests in the mothers of these patients substantiates the evidence that the infection was acquired in utero. In at least one instance, however, the blood of a child with encephalitis was positive while the mother's blood was negative, indicating that the infection was probably acquired postnatally. It is not known to what extent the presence of antibodies means past infection. Indeed, rough estimates place the incidence of neutralization from 2 per cent to about 10 per cent in different regions.[1, 10, 11] Of greater importance is the constant association of a certain syndrome with the presence of neutralizing antibodies. In this case the problem becomes one of statistical study. Such studies have already demonstrated the close association between positive sera in young children presenting the cardinal signs of congenital toxoplasmosis on the one hand and the presence of antibodies in the mothers' blood on the other. Occasionally other members of the immediate family have had circulating antibodies against toxoplasma, giving credence to the belief that the infant acquired the infection in utero at a time when the infection was widespread among some members of the family.

Although no exact figures are available, it would appear that antibodies once attained as a result of specific infection persist for long periods of time, perhaps indefinitely. The longest period of examination was until about five years after diagnosis, at which time the serum neutralized to the same extent as before. Numerous lesser intervals have revealed that the antibodies were still present in the blood stream. For example, among the ninety tests performed on seventy-two individuals, some repeat examinations with freshly drawn serum samples were included. The specimens were obtained anywhere from two weeks to many months after the initial bleeding tests were performed. Among the twenty-two individuals with positive sera, nine were retested, of which eight remained positive and one became equivocal. Among the fifty negatives, five were retested and all remained negative. Seven equivocal individuals were found and of these, four were re-examined at a later date. One remained doubtful, two became negative, and one turned positive. This last occurred in an infant suspected of toxoplasmosis, whose test was equivocal at one month of age but became strongly positive at three months of age. This observation has been pointed out by Sabin[4] and to a certain extent explains the negative and equivocal results obtained by others in an occasional infant with toxoplasmosis. It does not explain the observation by Heidelman[11] of individuals who neutralized and subsequently lost their antibodies. A suggested explanation is that the sera originally were actually equivocal, since we have recorded only one instance in which a positive individual eventually became negative. Even this individual was originally on the borderline of positive and later became

equivocal. We thus far have not seen a strongly positive serum that later became completely negative.

Negative tests were obtained in many of the disease conditions and the small selection reported here emphasizes the need of tests on additional diseases of obscure etiology to rule out the possibility of toxoplasmosis. Such tests[1] have already excluded the possibility of toxoplasmic infection in patients with hydrocephalus, microcephaly, psychomotor retardation, or convulsions not associated with cerebral calcification or chorioretinitis or both. All of the sera from ten patients with hydrocephalus or microcephaly in the absence of cerebral calification or chorioretinitis were negative for neutralizing antibody. These, together with the sera from eight of nine patients reported here, make a total of eighteen of nineteen patients tested that failed to show antibodies when neither cerebral calcification nor chorioretinitis was present.

SUMMARY

The toxoplasma neutralization test performed in rabbits was used to determine the presence of antibodies in various disease conditions. Of the sera obtained from seventy-two selected individuals, twenty were positive, forty-eight were negative, and four were equivocal. Corroboration was obtained of the high incidence of the positive sera among children showing signs of congenitally acquired toxoplasmosis, namely, convulsions or other signs of central nervous system involvement and hydrocephalus or microcephalus when these were associated with cerebral calcification or chorioretinitis or both. Antibodies were regularly present in the blood of the mothers of such patients. Antibodies were also found in a child with prolonged encephalitis and in two adults with mental disturbances. The toxoplasma neutralizing antibodies persist in the blood stream for at least five years, which was the longest interval tested. Many conditions of obscure etiology failed to show the presence of toxoplasma antibodies.

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REFERENCES

1. Sabin, A. B.: Toxoplasma Neutralizing Antibody in Human Beings and Morbid Conditions Associated With It, Proc. Soc. Exper. Biol. & Med. **51**: 6, 1942.
2. Johansmann, R. J., and Ruchman, I.: To be published.
3. Miller, M. C.: Infantile Toxoplasmosis, J. Pediat. **30**: 201, 1947.
4. Sabin, A. B.: Toxoplasmosis: a Recently Recognized Disease of Human Beings. Advances in Pediatrics, vol. 1, New York, 1942, Interscience Publishers, Inc., p. 1.
5. Sabin, A. B.: Toxoplasmic Encephalitis in Children, J. A. M. A. **116**: 801, 1941.

6. Sabin, A. B., and Olitsky, P. K.: Toxoplasma and Obligate Intracellular Parasitism, Science **85**: 336, 1937.
7. Cowen, D., Wolf, A., and Paige, B. H.: Toxoplasmic Encephalomyelitis: Clinical Diagnosis of Infantile or Congenital Toxoplasmosis; Survival Beyond Infancy, Arch. Neurol. & Psychiat. **48**: 689, 1942.
8. Sabin, A. B., and Ruchman, I.: Characteristics of the Toxoplasma Neutralizing Antibody, Proc. Soc. Exper. Biol. & Med. **51**: 1, 1942.
9. Vail, D., Strong, J. C., and Stephenson, W. V.: Chorioretinitis Associated With Positive Serological Tests for Toxoplasma in Older Children and Adults, Am. J. Ophth. **26**: 133, 1943.
10. Callahan, W. P., Jr.: Incidence of Toxoplasmic Infections in the St. Louis Area, Proc. Soc. Exper. Biol. & Med. **59**: 68, 1945.
11. Heidelman, J. M.: Evaluation of Toxoplasma Neutralization Tests in Cases of Chorioretinitis, Arch. Ophth. **34**: 28, 1945.
12. Adams, F. H., Horns, H., and Eklund, C.: Toxoplasmosis in a Large Minnesota Family, J. Pediat. **28**: 165, 1946.
13. Johnson, L. V.: Use of Neutralizing Antibody Test in Diagnosis of Human Toxoplasmic Choroiditis, Arch. Ophth. **36**: 677, 1946.

LABORATORY METHODS

A COLORIMETRIC DETERMINATION OF CARONAMIDE

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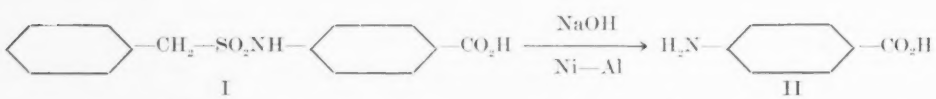
THE evaluation of a large number of substances of various chemical types for their property of inhibiting the renal elimination of penicillin has led to the selection of 4′-carboxy-phenylmethanesulfonanilide (caronamide) for detailed study.[1, 2] The extensive pharmacological and clinical evaluation of such an agent requires a method for the quantitative determination of the substance in tissues and in body fluids. The convenience and acceptability of the colorimetric method for the quantitative determination of sulfanilamide derivatives[3, 4] led to attempts to adapt this method for the determination of caronamide. Since caronamide (I) is a derivative of p-aminobenzoic acid, a cleavage to yield p-aminobenzoic acid (II) would permit the application of the diazotization method to this compound. This general colorimetric procedure has been employed for the determination of p-aminobenzoic acid and its derivatives in biologic material.[5, 6]



I II

Caronamide proved to be resistant to hydrolytic cleavage except under drastic conditions that resulted in the destruction of the p-aminobenzoic acid also. However, it was found that the action of a powdered nickel-aluminum alloy (Raney catalyst alloy*) in alkaline solution gave complete and smooth cleavage. In this method the reaction of the aluminum in the alloy with the sodium hydroxide results in a vigorous evolution of hydrogen. The hydrogen, in contact with the nickel from the alloy, causes hydrogenolysis of the caronamide with the liberation of p-aminobenzoic acid which then may be determined by the established procedures.

A description of the procedures for the determination of caronamide in urine, in plasma, and in blood is presented in this communication. The application of this method to other compounds will be reported later.

In the procedures for plasma and blood, 90 per cent alcohol proved to be a satisfactory protein precipitant. Acid precipitants such as trichloroacetic acid or p-toluenesulfonic acid were not satisfactory because of the low solubility of caronamide in acid media and because of the large volume obtained when the

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acid filtrate was neutralized and then made sufficiently alkaline for treatment with the alloy. In the case of plasma it is not essential that the protein be precipitated. In the alternative procedure the plasma in alkaline solution is treated directly with the alloy.

REAGENTS

Sodium hydroxide solution, approximately 5 per cent

Raney catalyst alloy

Hydrochloric acid, approximately 6 N; the concentrated acid diluted with an equal volume of water

n-Octyl alcohol, to prevent foaming with alloy treatment

Sodium nitrite, 0.2 per cent solution, freshly prepared

Ammonium sulfamate, 2 per cent solution

N-(1-Naphthyl)-ethylenediamine dihydrochloride, 0.1 per cent solution, freshly prepared

Ethanol, 90 per cent

PROCEDURES

For the determination of the p-aminobenzoic acid following the alloy treatment, a slight modification of the procedure described by Eckert[5] is used. N-(1-Naphthyl)-ethylenediamine is employed as the coupling agent. A Klett-Summerson photoelectric colorimeter with a No. 54 green filter was used to read color intensities.[5, 6]

I. Determination in Urine.—Two milliliters of the urine sample are diluted accurately to 10 ml. with water.* One milliliter of this solution is added to 10 ml. of 5 per cent sodium hydroxide solution in a 125 ml. flask or a large test tube. Two drops of n-octyl alcohol are added and then 0.5 Gm. of the Raney catalyst alloy.† The vessel is swirled gently to mix the contents. When the vigorous reaction has subsided, the mixture is heated on a steam bath or in a boiling water bath for twenty minutes. The mixture is cooled, and the residual nickel allowed to settle. The aqueous portion is decanted from the nickel and the nickel is washed by decantation four times with distilled water. The aqueous solution and the washings are combined and diluted to 100 milliliters. Instead of washing by decantation, the residual alloy may be separated by filtration or centrifugation‡ and the solution made up to 100 milliliters. Ten milliliters of the alkaline solution are added to 4 ml. of 6 N hydrochloric acid§ with constant agitation and the acid solution is diluted to 50 milliliters.

To 10 ml. of the acidified solution, 1 ml. of 0.2 per cent sodium nitrite solution is added. The solution is mixed well and allowed to stand for five minutes. One milliliter of 2 per cent ammonium sulfamate solution is added; the solution is mixed and allowed to stand for three minutes. Finally, 1 ml. of the 0.1 per cent solution of N-(1-naphthyl)-ethylenediamine dihydrochloride is added and the solution is mixed. After thirty minutes the intensity of the color is read. Distilled water is used as a blank. The concentration of caronamide in the original urine sample is obtained by reference to a standard curve constructed by plotting colorimeter readings obtained when a series of standard solutions of drug is carried through the foregoing procedure.

II. Determination in Plasma.—A 2 ml. sample of plasma is diluted accurately to 20 ml. with 90 per cent alcohol and the suspension is thoroughly mixed. After standing for

*This primary dilution is desirable to minimize foaming during the alloy treatment and to reduce the concentration of caronamide.

†The alloy may be measured conveniently in a small measuring spoon that is shaped to hold approximately 0.5 gram.

‡Caution: The residual nickel must not be allowed to dry since it is pyrophoric. It should be washed down the drain immediately with a large quantity of water.

§The alkaline solution must be added to the acid. If the addition is in the reverse order a precipitate forms which dissolves rapidly in the excess acid only when the mixture is warmed.

ten minutes the mixture is filtered through coarse paper and 10 ml. of the filtrate are added to 10 ml. of 5 per cent sodium hydroxide solution. Two drops of n-octyl alcohol are added, followed by 0.5 Gm. of the Raney alloy.* When the first vigorous reaction subsides, the mixture is heated on a steam bath or in a boiling water bath for thirty minutes. The alcohol is allowed to escape during this treatment. The mixture then is cooled, diluted to 100 ml., and filtered.† Ten milliliters of the filtrate are added to 1 ml. of 6 N hydrochloric acid.‡ After the addition of 1 ml. of 0.2 per cent sodium nitrite, the solution is mixed well and allowed to stand for five minutes. One milliliter of 2 per cent ammonium sulfamate is added. After three minutes, 1 ml. of 0.1 per cent N-(1-naphthyl)-ethylenediamine dihydrochloride solution is added and the color is allowed to develop for thirty minutes. The color intensity is read, using distilled water as the blank.

The concentrations of caronamide are calculated by reference to a standard curve prepared by plotting colorimeter readings obtained by using this procedure on samples of plasma containing known quantities of the drug.

III. Alternative Determination in Plasma.—A 1 ml. sample of plasma is diluted with 10 ml. of 5 per cent sodium hydroxide solution. To this are added two drops of n-octyl alcohol and 0.5 Gm. of Raney alloy. When the first vigorous reaction has subsided, the reaction mixture is heated on the steam bath for thirty minutes. The mixture then is cooled, diluted to 100 ml., and filtered.†

A 10 ml. aliquot of the filtrate is added to 1 ml. of 6 N hydrochloric acid‡; after which 1 ml. of the 0.2 per cent sodium nitrite solution is added. This is mixed well and allowed to stand for five minutes. One milliliter of 2 per cent ammonium sulfamate is added. After the solution has stood for three minutes, 1 ml. of 0.1 per cent N-(1-naphthyl)-ethylenediamine dihydrochloride solution is added and the color is allowed to develop for thirty minutes. Intensity of color is read with the colorimeter. In order to correct for a slight turbidity that appears with some plasma specimens, a blank is prepared from a second 10 ml. sample of the alkaline filtrate from the alloy treatment by omitting the sodium nitrite.

Concentrations of caronamide are calculated from a standard curve prepared by plotting colorimeter readings obtained by using the foregoing procedure on samples of plasma containing known quantities of drug.

IV. Determination in Blood.—A 2 ml. sample of blood is added to 2 ml. of water and the mixture is allowed to stand for ten minutes in order to allow hemolysis of the cells. This mixture is diluted accurately to 20 ml. with 90 per cent alcohol. The suspension is mixed thoroughly, allowed to stand for ten minutes, and filtered. Ten milliliters of the filtrate are carried through the alloy treatment as described in II. A standard curve is constructed from values obtained with blood to which known amounts of the drug have been added.

Results With Standard Solutions.—Solutions containing known amounts of p-aminobenzoic acid in water, in urine, in plasma, and in blood were prepared and run through the alloy treatment according to these procedures. The amounts of p-aminobenzoic acid recovered were calculated by reference to a plot of the colorimeter readings that were obtained on standard aqueous solutions not subjected to the alloy treatment. The results, which are tabulated in Table I, show that recoveries generally were 80 to 90 per cent. Therefore, 10 to 20 per cent of the p-aminobenzoic acid is lost during the alloy treatment. However, for each procedure the recoveries are fairly constant and reproducible.

Similarly, solutions containing known amounts of caronamide were subjected to these procedures and the recoveries of p-aminobenzoic acid were cal-

*See footnote † on page 97.
†See footnote ‡ on page 97.
‡See footnote § on page 97.

TABLE I. RECOVERY OF P-AMINOBENZOIC ACID

PROCEDURE	PAB ADDED (MG./100 ML.)	PAB RECOVERED	
		MG./100 ML.	%
	In Water		
I	500	424	85
I	250	206	82
I	100	84	84
III	20	17	85
III	10	8.3	83
III	5	4.2	84
	In Urine		
I	500	456	91
I	250	228	91
I	100	90	90
	In Plasma		
II	20	16.6	83
II	10	8.6	86
II	5	4.3	86
III	20	16	80
III	10	8	80
III	5	4	80
	In Blood		
IV	20	15.6	78
IV	10	8.2	82
IV	5	4.1	82

culated. Again the recoveries were generally between 80 and 90 per cent and were constant and reproducible for each procedure. Since the recoveries were essentially the same after the alloy treatment of either p-aminobenzoic acid or caronamide, it appears that the caronamide is split quantitatively but that an average of 15 per cent of the liberated p-aminobenzoic acid either is destroyed during the treatment or is lost, possibly by adsorption on the finely divided nickel.

TABLE II. RECOVERY OF P-AMINOBENZOIC ACID FROM CARONAMIDE

PROCEDURE	CARONAMIDE ADDED (MG./100 ML.)	PAB EQUIVALENT (MG./100 ML.)	PAB RECOVERED	
			(MG./100 ML.)	%
	In Water			
I	1000	470	405	86
I	500	235	202	86
I	200	94	84	89
	In Urine			
I	1000	470	408	87
I	500	235	205	87
I	200	94	84	89
	In Plasma			
II	40	18.8	16.4	87
II	20	9.4	8.3	88
II	10	4.7	4.1	87
III	40	18.8	14.4	76
III	20	9.4	7.2	76
III	10	4.7	3.2	68
	In Blood			
IV	40	18.8	15.4	82
IV	20	9.4	7.8	83
IV	10	4.7	4.0	85

From these data it is evident that standard reference curves for use in routine determinations may be constructed from the colorimeter readings obtained when urine, plasma, or blood containing known amounts of caronamide are run through the appropriate procedure. From Table I and Table II it will be noted that procedure III, which is run on plasma without removal of protein, gives lower recoveries than does procedure II. Nevertheless, this procedure has proved to be useful in the analyses of a large number of routine specimens where extreme accuracy was not required.

For the best results the acid solutions used for the diazotization and coupling in these procedures should correspond to a caronamide concentration of between 0.15 and 0.4 mg. per 100 ml., or approximately 0.075 to 0.2 mg. per 100 ml. of p-aminobenzoic acid. At these concentrations the scale readings of the colorimeter lie between 100 and 300.

With the dilutions employed in these procedures (1:2,500 for urine, and 1:100 for plasma and blood), caronamide concentrations in urine from 200 to 1,000 mg. per 100 ml., and in plasma or blood from 5 to 50 mg. per 100 ml., can be determined satisfactorily.

SUMMARY

A procedure for the colorimetric assay of caronamide is desribed. Treatment in aqueous alkali with nickel-aluminum alloy quantitatively liberates p-aminobenzoic acid which then is determined by established procedures.

REFERENCES

1. Beyer, K. H.: New Concept of Competitive Inhibition of the Renal Tubular Excretion of Penicillin, Science **105**: 94, 1947.
2. Beyer, K. H., Miller, Katherine, Russo, Horace F., Patch, Elizabeth A., and Verwey, Willard F.: The Inhibitory Effect of Caronamide on the Renal Elimination of Penicillin, Am. J. Physiol. **149**: 355, 1947.
3. Marshall, E. K.: Determination of Sulfanilamide in Blood and Urine, J. Biol. Chem. **122**: 263, 1937-1938.
4. Bratton, A. Calvin, and Marshall, E. K.: A New Coupling Component for Sulfanilamide Determination, J. Biol. Chem. **128**: 537, 1939.
5. Eckert, H. William: Determination of p-Aminobenzoic Acid, Conjugated p-Aminobenzoic Acid and p-Nitrobenzoic Acid in Blood, J. Biol. Chem. **148**: 197, 1943.
6. Kirch, Ernest R., and Bergeim, Olaf: Determination of p-Aminobenzoic Acid, J. Biol. Chem. **148**: 445, 1943.

A COMPENSATING PLETHYSMOKYMOGRAPH FOR MEASURING BLOOD FLOW IN HUMAN EXTREMITIES*

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ACCURATE measurement of blood flow through the extremities of human beings has proved to be a fairly difficult problem. That plethysmokymographic methods are not completely satisfactory is attested by the fact that for the past thirty years new improvements of the original plethysmokymograph invented by Brodie and Russell[1] have been presented frequently. The skepticism of Pezzali[2] concerning the value of this method for measuring blood flow has frequently proved to be justified although the plethysmokymograph has yielded a large volume of valuable physiologic information of qualitative if not quantitative significance. Unfortunately, however, most of the physical and physiologic principles on which the method is based have never been adequately analyzed. In fact, it has been demonstrated in only one organ[3] (the kidney) that blood flow is measured by the plethysmokymographic method. Landowne and Katz[4] have presented an admirable critique of the plethysmographic method of measuring blood flow in human beings.

PRINCIPLE OF PLETHYSMOKYMOGRAPHY

The term plethysmokymography is derived from Greek and literally means "the recording of the curve of filling." An organ or a portion of an extremity is sealed in a leakproof plethysmograph. A syphygmomanometer cuff connected to a pressure reservoir is placed about the extremity of the subject just proximal to the plethysmograph. This cuff is called the collecting cuff and its function is to produce occlusion of the veins. Sudden inflation of the collecting cuff to a pressure below diastolic blood pressure, yet above venous pressure, traps incoming arterial blood within the portion of the extremity distal to the collecting cuff and thereby causes engorgement and increase in volume of the portion of the extremity which lies inside the plethysmograph. Connected by tubing to the inside of the plethysmograph is a recording device which is activated by a change of pressure within the plethysmograph. This change of pressure within the plethysmograph and recorder is induced by a change in volume of the enclosed portion of an extremity. The recorder may trace on a smoked drum or reflect a beam of light to record on moving photosensitive paper. The latter method is called the optical system of recording and is the more satisfactory of the two

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when sensitivity is essential. The rate of increase in volume of the portion of the extremity enclosed in the plethysmograph is measured by the excursion of the recorder and a timing device. The increase in volume per unit of time, so obtained, is assumed to be an indication of actual blood flow to the portion of the extremity enclosed in the plethysmograph. The record obtained is called a "flow curve." Fig. 1 shows several types of flow curves which were obtained in this study.

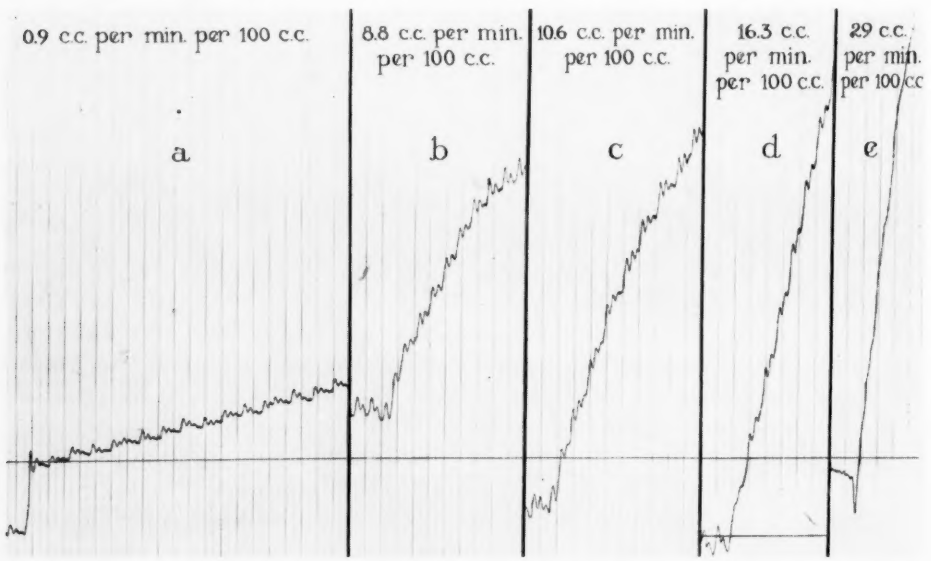

Fig. 1.—Typical blood flow curves obtained from the arms of human beings by the plethysmographic method. Each record was taken on a different subject. Calculated volume flow for each curve is given at the top of the respective tracing.

The purpose of this communication is to describe a compensating plethysmokymographic method which we have developed. After careful analysis and appraisal of this and various other methods we have reached the conclusion that this compensating plethysmokymograph most nearly satisfies the criteria for accurate determination of blood flow in human extremities. The following are the main requirements which we consider essential for the accurate determination of blood flow through human extremities by the plethysmokymographic method:

1. Accurate measurement of pulsating changes in volume per second
2. Approximately normal environmental conditions
3. Complete and sudden arrest of the return of venous blood from the extremity without obstruction of the flow of arterial blood into the extremity
4. Elimination of or compensation for mechanical errors due to:
   (a) Displacement of fluid and tissue from beneath the collecting cuff into the plethysmograph during inflation of the collecting cuff
   (b) Respiratory and other motions of the extremity
   (c) Differences in calibration of the recording device due to the differences in the volume of various extremities

(d) Changes in environmental temperature and pressure
(e) Leaks in the apparatus
(f) Slight variations in camera speed.

Air was preferred to water as an environmental medium inside the plethysmograph for the following reasons:

1. Water constitutes an abnormal environmental medium.

2. The inertia of water might tend to slow down rapid changes in volume of an extremity.

3. External water pressure may prevent increases in volume of the venous system.

4. We desired a method of plethysmokymography which would be applicable to the leg and foot as well as to the arm and hand. With water as an environmental medium, the patient's knee must be at a higher level than the foot in order to prevent leakage of water about the point where the plethysmograph is sealed to the patient's extremity. If the patient's foot is at a lower level than his knee, venous pressure and the external pressure of the surrounding water are greater in the foot than in the leg and arterial blood should flow into the leg more easily than into the foot.

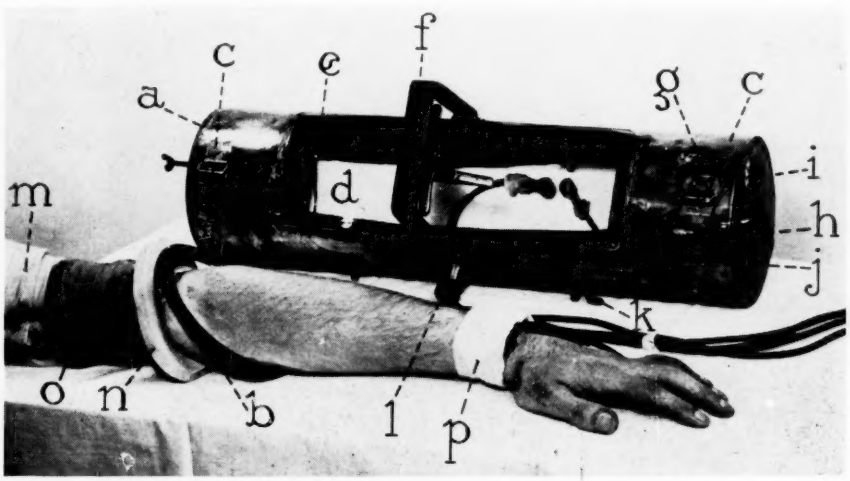

Fig. 2.—The arm plethysmograph with the arm prepared for insertion into the plethysmograph, *a*, Open end of the plethysmograph; *b*, brass ring for clamping the occluding diaphragm to the open end of the plethysmograph (this is passed over the diaphragm before clamping); *c*, metal straps for swinging the plethysmograph from above; *d*, plate-glass window resting on rubber gasket; *e*, rubber gasket; *f*, spring clamp holding the window in place; *g*, inlet for calibrator; *h*, outlet for a Marey tambour; *i*, outlet for wide-bore tubing leading to the compensating spirometer recorder; *j*, outlet to the recorder for a finger plethysmograph, also used as the inlet for perfusing the plethysmograph when calibrating; *k*, inlet for inflation of the wrist cuff; *l*, inlet for four electric thermocouples; *m*, rubber sleeve; *n*, rubber sleeve everted over a sponge rubber occluding diaphragm; *o*, collecting cuff; *p*, wrist cuff.

DESCRIPTION OF APPARATUS*

Plethysmograph (Fig. 2).—A copper cylinder 18 inches (45.7 cm.) long and 6 inches (15.2 cm.) in diameter is closed at one end and open at the other. In the side of the cylinder is a rectangular opening 4 by 8 inches (10.2 by 20.3 cm.) in diameter. A plate-glass window held in place by a strong spring clamp

*We are indebted to Mr. A. N. Porter for his technical assistance.

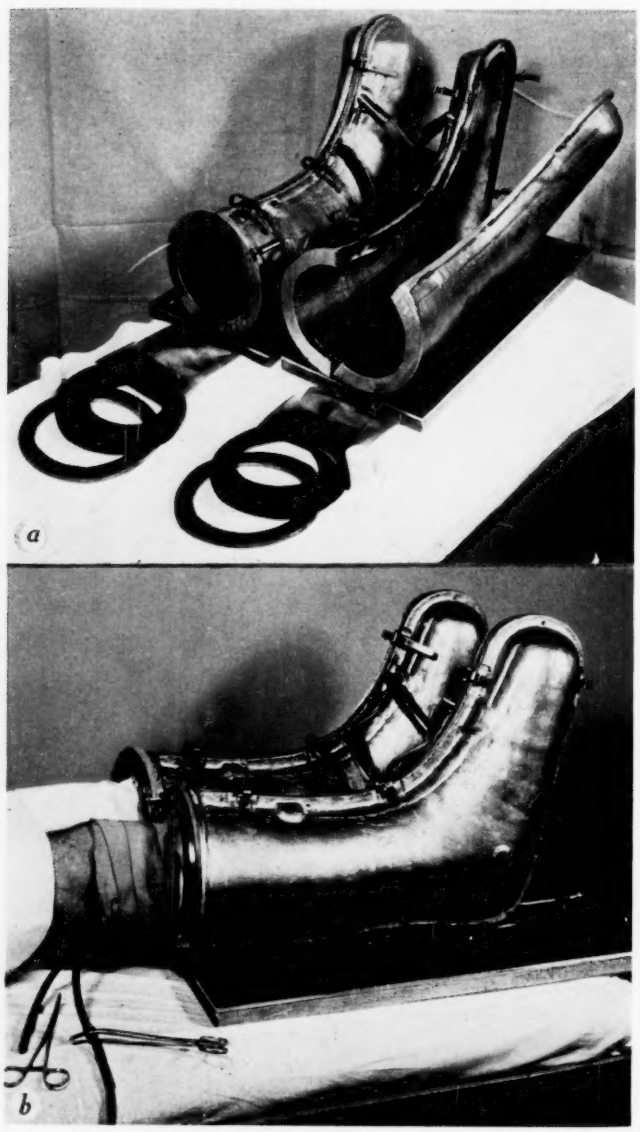

Fig. 3.—Leg plethysmograph showing the various stages in the preparation of the leg for making an airtight seal around the leg when inserted into the plethysmograph without interfering with the circulation. The various outlets corresponding to those of the arm plethysmograph are located on the medial surfaces. *a*, Constituent parts of leg plethysmograph; *b*, plethysmograph applied to legs and made airtight without interference with circulation.

rests on a sponge rubber gasket which occludes the juncture between the edge of the rectangular window and the plate glass. When the plethysmograph is horizontal, the window is on the upper side and permits visualization of the limb. On the right side of the plethysmograph is a round opening large enough to convey the lead wires of electric thermocouples for determination of skin temperatures. Outlets for a Marey tambour and a calibrator are placed at the top of the distal end of the plethysmograph. On the side of the plethysmograph is a

connection for inflating a wrist cuff. On the distal end is an outlet for a recorder connected to a finger plethysmograph and another outlet for a recorder connected to the arm plethysmograph. The instrument is suspended and swung by means of a rope from a point above and moves freely from side to side or back and forth with respiratory and other involuntary motions of the extremities. The plethysmograph is rendered airtight by means of a thick, stiff sponge rubber diaphragm called the occluding diaphragm which is clamped over the open end of the plethysmograph by a heavy brass ring.

Plethysmographs for the lower extremities were made of cast aluminum as shown in Fig. 3. The major principle of their operation is the same as for the arm plethysmographs described previously. The plethysmographs were painted flat black in order to facilitate heat transfer and prevent abnormal variations of temperature.

Blank Plethysmograph.—The blank plethysmograph is a replica of the afore-mentioned plethysmograph and is used in conjunction with compensating spirometers (see next section) to eliminate the errors induced in the estimation of blood flow by changes in the environmental air temperature and pressure.

Compensating Spirometers (Fig. 4).—The recording device, which we have named a compensating spirometer, is made up of two spirometers identical in capacity and form. Each spirometer is made of thin shim brass in the shape of a hollow, truncated wedge. One of the broad sides of the wedge is omitted, thus forming an inverted cup when the wedge is placed with the open side down. Each spirometer rotates about an axle mounted on ball bearings. The axles of the two spirometers lie in the same axis. Each spirometer floats on kerosene or "finol" contained in a square metal pan. A metal pipe pierces the bottom of the pan passes up through the contained kerosene and opens into the air pocket in the spirometer. Wide-bore rubber or plastic tubing connects one spirometer to the plethysmograph and the other to the blank plethysmograph. In this way the enclosed space between the top of each spirometer and the surface of the kerosene is in communication with the respective plethysmograph. As the intraplethysmographic volume increases the spirometer rises; as the volume decreases it falls. The spirometer pans are mounted side by side and 1 cm. apart. An extension of the axle of each spirometer projects into the space between them and to each extension a small mirror is cemented.

The two spirometers are so placed that a single-filament lamp throws a horizontal beam of light through the space separating the spirometer pans and into the camera. The mirror mounted on the axle of the spirometer connected to the blank plethysmograph reflects the beam of light from the lamp backward and onto the mirror attached to the spirometer connected to the true plethysmograph. The latter mirror reflects the beam of light forward through a focusing lens into the camera. Thus the optical system is so arranged that an increase in volume of the blank plethysmograph causes a downward deflection of the recording beam while an increase in volume of the true plethysmograph causes an upward deflection of the light beam. Any factor which changes the volume of both the true plethysmograph and the blank plethysmograph simultaneously to the same degree and in the same direction does not appreciably

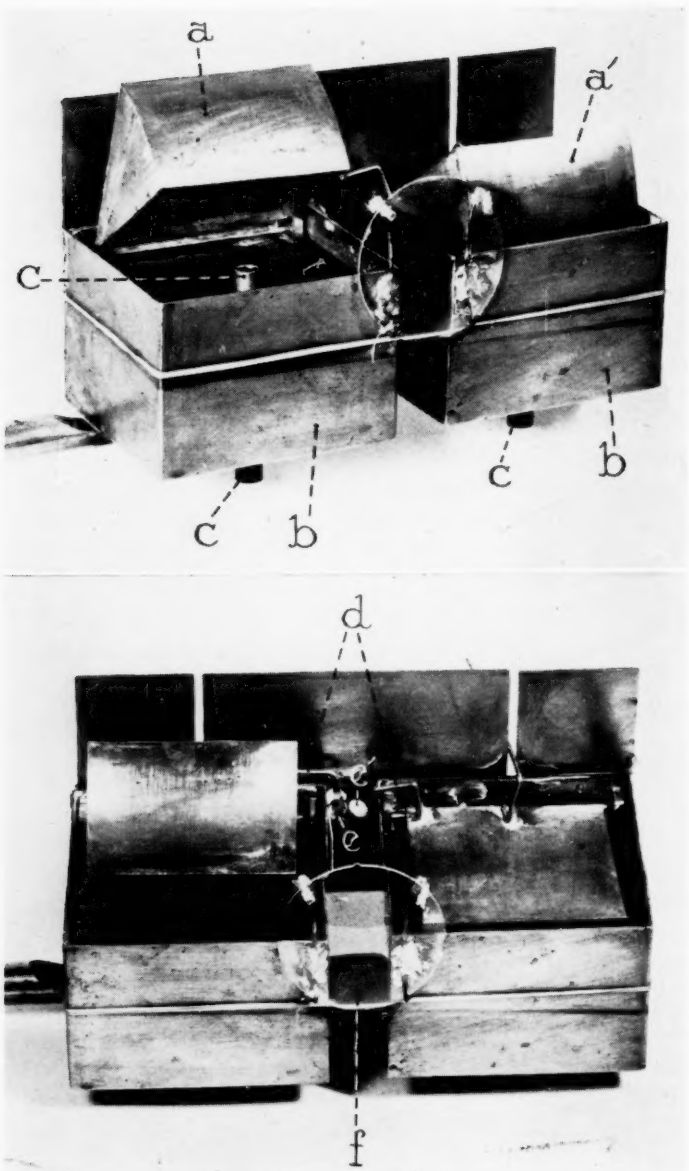

Fig. 4.—Compensating spirometer recorder. *a*, Spirometer for recording volume changes in the blank plethysmograph; *a'*, spirometer for recording volume changes in the "true" plethysmograph; *b*, metal pans containing "finol" which seals the spirometers; *c*, metal pipes piercing the bottoms of the pans for connection with the plethysmographs; *d*, extension of the axles of the spirometers into the space between the pans; *e*, mirror facing backward; *e'*, mirror facing forward; *f*, focusing lens.

alter the position of the recording beam. Since changes in room temperature and pressure affect the true plethysmograph and the blank plethysmograph equally and simultaneously, the errors caused by these changes are eliminated from the flow curve (Fig. 5).

The mechanism for compensation is also demonstrated in Fig. 6. If venous occlusion is made in both extremities simultaneously, the deflection of the beam

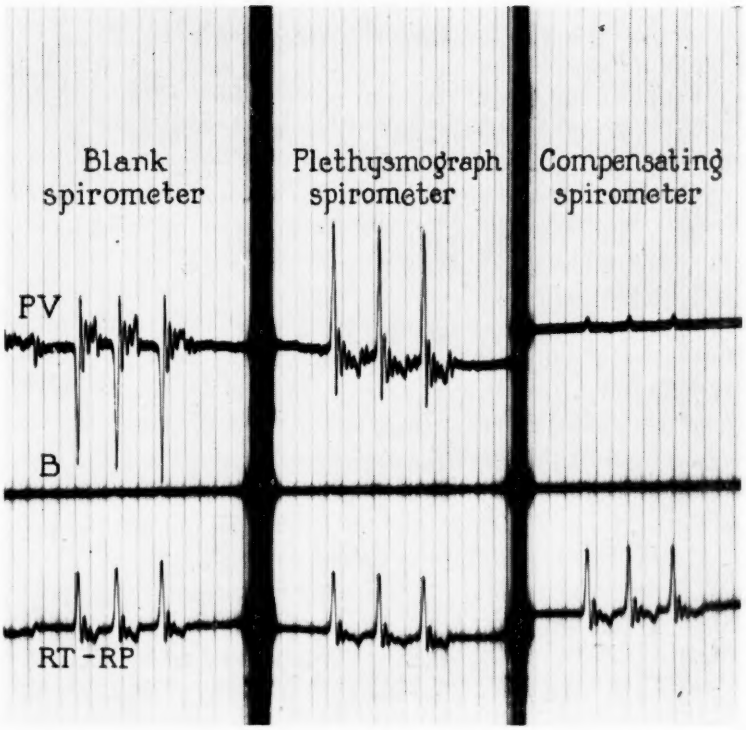

Fig. 5.—Tracings illustrating the mechanism by means of which compensating spirometers compensate for fluctuations in room temperature and pressure. *PV*, Plethysmograph volume; *B*, base line; *RT-RP*, room temperature and room atmospheric pressure.

Fluctuations in atmospheric pressure were induced by suddenly opening and closing the door of the laboratory three times during each experiment. Such fluctuations are recorded in the *RT-RP* line. Note that they are about equal in each of the three experiments.

Blank spirometer: Tube leading to the plethysmograph was clamped so that only the spirometer recording volume changes in the blank plethysmograph recorded. Note that the major deflection of the spirometer is downward.

Plethysmograph spirometer: Tube leading to the blank plethysmograph spirometer was clamped so that only the plethysmograph spirometer recorded. The major deflection is now upward.

Compensating spirometers: Both the blank and plethysmograph spirometers recorded. The downward deflection of the former is neutralized by the upward deflection of the latter and no significant deflection of the recording beam occurs despite changes in atmospheric pressure.

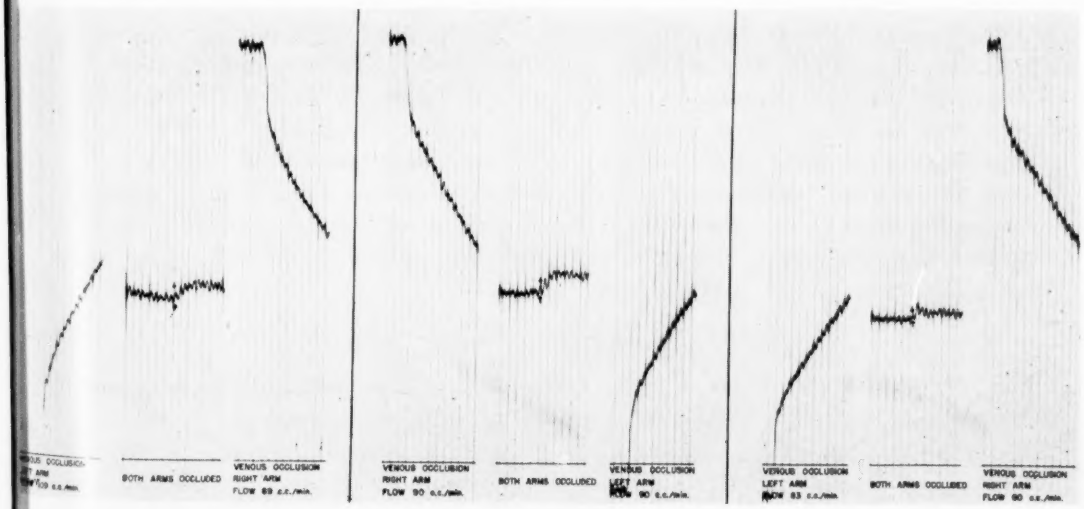

Fig. 6.—Tracings demonstrating the mechanism of compensation by use of compensating spirometer recorder. Blood flow curves were taken from the left and right forearms of the same individual. Simultaneous venous occlusion of both arms caused only an insignificant deflection of the beam.

is insignificant; yet venous occlusion of the left or right limb alone will give a typical flow curve for that limb. It is clearly demonstrated that the deflection of the light beam by occlusion of the right arm is downward and its base line had to be made at the top of the camera slit. Venous occlusion of the left arm, however, deflects the beam in a direction diametrically opposite to that of the right and its base line, therefore, is made at the bottom of the camera slit. Both flow curves for the right and left forearms can be made separately and calculated as shown in Fig. 6. Simultaneous occlusion of both arms produces practically identical changes in volume in both arms at the same time and therefore the upward deflection of the beam produced by one arm annuls the downward deflection produced by the other. Hence, by the use of compensating spirometers simultaneous changes in the limbs similar in magnitude and direction are taken care of without significant error.

Marey Tambour.—A Marey tambour with an optical system of recording is used to test for the presence of leaks. To test for leaks, the tube leading to the compensating spirometer from the plethysmograph is clamped. Enough air is injected into the plethysmograph to induce a pressure of 1.5 cm. of water and the deflection of the beam of light reflected from the rubber tambour is noted. A persistent fall in pressure is indicative of a leak in the apparatus.

PROCEDURE FOR MAKING AN AIRTIGHT SEAL BETWEEN THE LIMB AND THE PLETHYSMOGRAPH WITHOUT INTERFERING WITH THE CIRCULATION

The procedure that was used for making an airtight seal consisted of coating the part of the limb proximal to the plethysmograph with surgical jelly before inserting it into a thin rubber sleeve 8 to 10 inches (20.3 to 25.4 cm.) long. The sleeve fits loosely around the limb (Fig. 2). A disk of sponge rubber ½ inch (1.3 cm.) thick and with a diameter equal to that of the inlet of the plethysmograph had a hole cut into it conforming to the contour of the limb but with a slightly greater circumference. The limb is slipped through the hole in the disk and the distal end of the rubber sleeve is everted over the rim of the disk. A bandage is wrapped snugly but lightly over the rubber sleeve and limb proximal to the disk. This helps to support the disk and sleeve, insure airtightness, and eliminate the presence of pockets under the sleeve. The distal surface of the rubber disk is coated with surgical jelly and clamped to the rim of the plethysmograph after insertion of the limb into proper position in the plethysmograph. The whole limb is then elevated sufficiently to insure venous drainage. An ordinary sphygmomanometer cuff used for children is wrapped around the limb about ½ inch (1.3 cm.) proximal to the occluding disk and is used as a collecting cuff.

PHYSICAL CHARACTERISTICS OF THE APPARATUS

Comparison of Various Types of Recorders.—In general, the various types of recorder used by previous investigators fall into two groups:

1. Those which maintain their deflection to a given increase of intraplethysmographic volume by means of pressure built up within the plethys-

mograph by the increment of volume. The Marey tambour and glass spoon manometer[5] are representative of this group. This group hereafter will be called pressure recorders.

2. Those which will tend to maintain their deflection without pressure, though a slight change in intraplethysmographic pressure is necessary to cause the initial deflection. Brodie bellows, piston recorders, and spirometers fall into this group. A single spirometer and compensating spirometers have been selected for study. This group hereafter will be called volume recorders.

The size of the tubing connecting the recorder to the plethysmograph should vary with the type of recorder. With a pure pressure recorder such as the glass spoon manometer, small-bore pressure tubing yields the best results. With volume recorders such as the compensating spirometers, large-bore tubing is essential. We have found tubing with an internal diameter of 1.5 cm. to be most satisfactory. Tubing of 1.00 cm. internal diameter or less introduces a definite lag in the recorder. Brodie[6] and Abramson, Zazeela, and Marrus[7, 8] also found wide-bore tubing essential when using volume recorders.

Frequency of the Recorders.—A Dale-Schuster pump was so arranged that 5 c.c. of air were injected into and extracted from the plethysmograph at the rate of about 50 cycles per second. The compensating spirometers failed to register these oscillations. The speed of the pump was then gradually decreased and it was found that the compensating spirometers responded to the alternate suction and pressure when these cycles occurred about twelve times per second. The pump was further slowed until the excursion of the compensating spirometers was maximal to each cycle. This occurred at 3.3 cycles per second and was taken to represent the natural frequency of the recorder. By the same method the natural frequency of a representative Marey tambour was 10 cycles per second, of a glass spoon manometer, 20 cycles per second, and of a single one of the compensating spirometers, 3.3 cycles per second.

The Effect on Various Recorders of Different Volumes of Air in the Plethysmograph.—The rubber tambour and glass spoon manometers gave increasingly higher deflections for the same increment of plethysmographic volume as the arm volumes increased. Single spirometers and the compensating spirometer gave practically identical deflections no matter what the intraplethysmographic volume of air might be.

When the volume of air in the plethysmograph-compensating spirometer unit was changed from 9,950 to 9,960 c.c., a deflection of 9 cm. occurred. When the volume was changed from 52 to 62 c.c., a deflection of 9.15 cm. occurred (Fig. 7).

Hence, if one uses a pressure recorder an individual calibration chart must be obtained for each subject investigated, since arm volume varies with each individual. On the other hand, with a volume type of recorder one may use the same calibration chart for any subject.

The Effect of Various Recorders on Pressure Within the Plethysmograph.—With a typical Marey tambour, slow injection of 10 c.c. of water into the plethysmograph induced an intraplethysmographic pressure of about 90 mm. of water.

Slightly greater pressures were induced by the same amount of water injected into the plethysmograph when a glass spoon manometer was used for recording. With the compensating spirometer a change of 0.5 mm. of water pressure will move the recorders appreciably. When properly balanced the compensating spirometer maintains a given deflection without appreciable change of pressure.

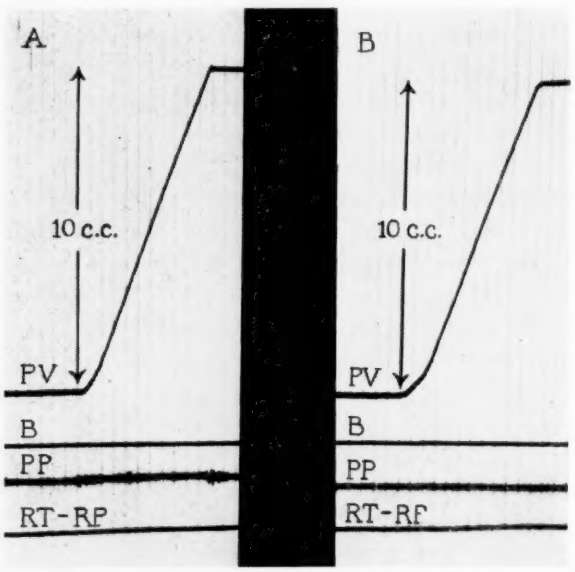

Fig. 7.—The effect of varying plethysmographic volume on the deflection of compensating spirometers. *PV*, Plethysmograph volume; *B*, base line; *PP*, pressure in the plethysmograph recorded by a Marey tambour; *RT-RP*, room temperature and room atmospheric pressure.

A, Volume of the plethysmograph-compensating spirometers unit changed from 9,950 c.c. to 9,960 c.c. by the injection of 10 c.c. of air into the plethysmograph. The deflection of the recorder was 9.0 centimeters.

B, Plethysmograph excluded from the plethysmograph-compensating spirometers unit leaving a volume of 52 cubic centimeters. The injection of 10 c.c. of air into the compensating spirometers induced a deflection of 9.15 centimeters.

The Effect on Various Recorders of Changing Environmental Pressure and Temperature.—Ellis,[9] in 1885, indicated that an air plethysmograph is very sensitive to changes of temperature. Turner,[10] in 1937, re-emphasized this point. We have found that all four types of recorders studied were very sensitive not only to changes in environmental temperature but also to air pressure. Single spirometers and Marey tambours were particularly vulnerable to errors induced by changes in environmental pressure or temperature. In fact it was impossible to obtain accurate flow curves on a windy day or in a place where doors were continually being opened and closed. On the other hand, the compensating spirometers eliminate errors induced by changes of room temperature or pressure, as discussed in describing the recorder (Fig. 5).

CALIBRATION OF THE PLETHYSMOKYMOGRAPH, USING THE COMPENSATING SPIROMETER FOR RECORDING

We thought it of interest to compare calibrations of the plethysmograph by the usual method of injecting known volumes of fluid into the plethysmograph and by a method whereby the plethysmograph is perfused with known

pulsating flows. By means of the Dale-Schuster pump one can deliver very nearly constant pulsating flows of amounts of fluid varying from 0 to 500 c.c. per minute. The pulse contour of the instrument simulates that of the heart. We have been unable to find any reference to the calibration of a plethysmograph by the use of known pulsating flows.

Method.—

1. *Injecting Known Volumes of Fluid Into the Plethysmograph by Means of a Syringe or Burette:* A section of rubber Penrose tubing was placed in the plethysmograph, extending through its entire length. The tube was clamped at its distal end. The other end opened to an outlet in the proximal end of the plethysmograph where it was connected to the delivery end of a certified 100 c.c. burette, accurate to 0.05 c.c., and filled with water. Increments of volume injected from the burette were then plotted against the actual deflections of the recording beam (Fig. 8, line *B*). Rapid injection from a syringe or slow injection from the burette gave the same calibration curve.

2. *Perfusing the Plethysmograph With Known Pulsating Flows:* The Penrose tubing was arranged in the same way inside the plethysmograph except that the end which had been clamped was fastened to an inlet through the distal end of the plethysmograph. A stopcock was inserted in the outlet of the Penrose tubing. Water was perfused through the tubing by means of a Dale-Schuster pump. The perfused water was measured in cubic centimeters per minute at least twice before and once after each flow curve was taken. The stopcock in the outlet was suddenly closed, thus trapping water within the Penrose tubing, and a tracing of the increase in intraplethysmographic volume per unit of time was obtained (Fig. 9). Flow curves of perfused flows ranging between 10 and 400 c.c. per minute were then obtained and the slope of each flow curve was drawn. Increments of volume of water collected were then plotted against actual deflections of the recording beam (Fig. 8, lines *A*, *C*, and *D*).

Method of Measuring Unknown Flows Produced by a Mechanical Schema.— Having obtained a calibration curve, one can deduce accurately the increase in volume within the plethysmograph from the resultant excursion of the recording beam. Unknown flows produced by a mechanical schema are measured as follows: The plethysmograph is perfused with an unknown pulsating flow delivered by a Dale-Schuster pump as described previously under Calibration of the Plethysmokymograph. The slopes of the flow curves obtained when compensating spirometers are used for recording are practically straight lines and therefore the flow may be calculated by any one of the following methods: (1) slope of the flow curve, (2) actual increment of volume per pulse beat, or (3) actual deflection of the recorder per unit of time. All methods give identical results within the experimental error of about 1 per cent. We have used calculations derived from the slope of the flow curve since this was found to be the most convenient method. The slope of the flow curve is drawn. A three-, six-, or ten-second interval of time is marked off along the base line of the flow curve from the point where the slope line intersects the base line. From the point so obtained, a perpendicular is erected to intersect the slope line. The length of

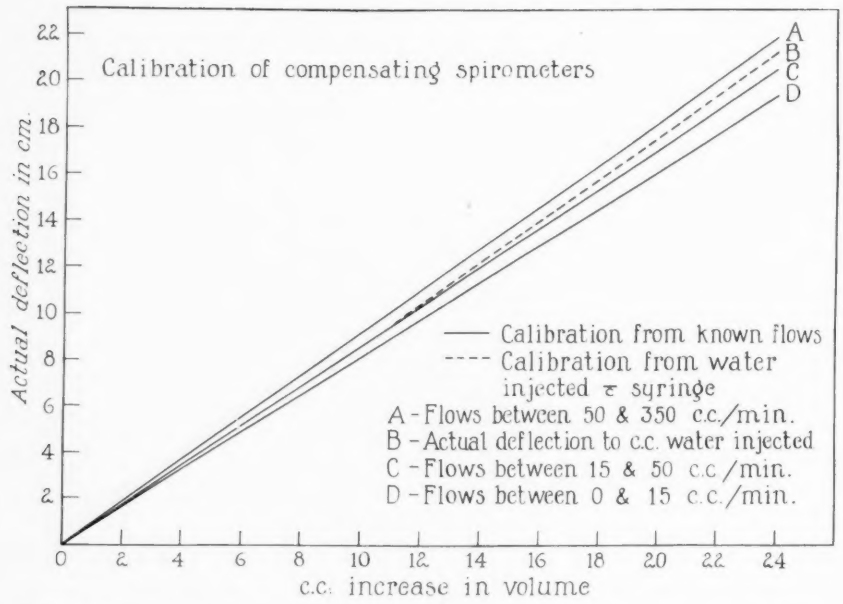

Fig. 8.—Calibration of compensating spirometers with flows of various magnitudes.

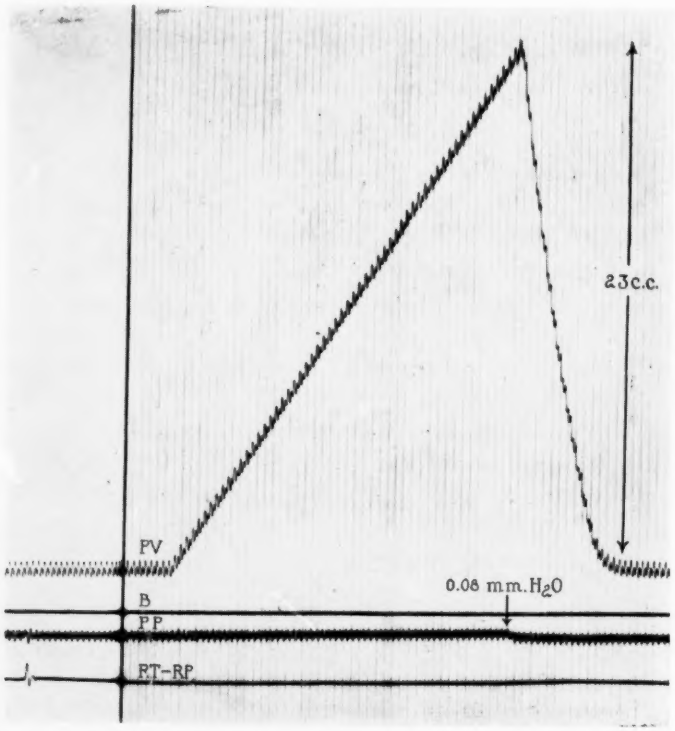

Fig. 9.—Typical flow curve obtained by perfusing the plethysmograph with known pulsating flows and using compensating spirometers for recording. *PV*, Plethysmographic volume recorded by compensating spirometers; *B*, base line; *PP*, plethysmographic pressure recorded by a Marey tambour (note that the maximal sustained pressure in the plethysmograph is 0.08 mm. of water); *RT-RP*, room temperature and room atmospheric pressure.

this perpendicular line represents the amount of air displaced from the plethysmograph into the recorder during the interval of time used. From the calibration curve, the length of the afore-mentioned perpendicular line may be easily converted to cubic centimeters of volume added to the plethysmograph per unit of time, and this figure then may be expressed as cubic centimeters of flow per minute.

Experimental Error of the Plethysmokymograph When a Mechanical Schema Was Used for Perfusion and the Compensating Spirometer Was Used for Recording.—Hewlett and Van Zwaluwenburg,[11] in 1909, estimated that their apparatus came within 20 per cent of measuring true blood flow in favorable cases. On the other hand, Stead and Kunkel,[12] in 1938, estimated the instru-

TABLE I. COMPARISON OF ACTUAL FLOW AND CALCULATED FLOW IN A MECHANICAL SCHEMA USING COMPENSATING SPIROMETERS FOR RECORDING

EXPERIMENT	ACTUAL FLOW (C.C. PER MIN.)	CALCULATED FLOW (C.C. PER MIN.)	PERCENTAGE ERROR OF CALCULATED FLOW
1	11.3	11.5	+1.8
2	12.5	12.5	0
3	12.5	12.5	0
4	12.5	12.5	0
5	13.0	13.0	0
6	17.0	17.0	0
7	17.5	17.0	-2.8
8	17.5	17.5	0
9	17.5	17.5	0
10	17.5	17.0	-2.8
11	17.7	18.0	+1.7
12	21.3	21.5	+0.9
13	45.0	40.5	-10.0*
14	68.0	68.0	0
15	68.0	68.0	0
16	70.5	69.0	-2.1
17	100.2	100.0	-0.2
18	116.5	119.0	+2.1
19	116.5	118.0	+1.3
20	117.5	119.0	+1.3
21	122.0	120.0	-1.6
22	140.0	138.0	-1.4
23	146.0	142.0	-2.7
24	146.0	145.0	-0.7
25	152.0	151.0	-0.6
26	155.0	150.0	-3.2
27	160.0	161.5	+0.9
28	160.0	155.0	-3.1
29	160.0	160.0	0
30	224.0	221.0	-1.3
31	224.0	220.0	-1.8
32	224.0	223.0	-0.4
33	224.0	220.0	-1.8
34	224.0	221.0	-1.3
35	282.0	278.5	-1.2
36	282.0	276.0	-2.1
37	282.0	279.5	-0.9
38	282.0	275.0	-2.5
39	340.0	339.0	-0.3
40	340.0	329.0	-3.2
41	340.0	337.5	-0.7
42	340.0	343.5	+1.0
43	340.0	339.0	-0.3
Average error			-0.88%

*No explanation could be found to account for the magnitude of this error.

mental error of their plethysmokymograph to be + or − 3 per cent. However, they found it necessary to add 13 per cent to the flows calculated from calibration curves obtained by the injection of air into the plethysmograph from a syringe. Prinzmetal and Wilson,[13] using an adaptation of Lewis and Grant's[14] instrument (1925), estimated the experimental error of the apparatus to be 15 per cent. Killian and Oclassen[15] estimated the experimental error of a modification of Hewlett and Van Zwaluwenburg's apparatus to be ± 15 per cent.

The compensating spirometer recorder was studied in detail concerning its accuracy in measuring flows within the usual limits of flow encountered in the human arm. The findings are shown in Table I. The person who calculated the flow from flow curves had no idea whatsoever as to what the actual flows were. The results may be summarized as follows: When compensating spirometers are used for recording pulsating flows produced by a mechanical schema, 98 per cent of the calculated flows lie between +2 and −4 per cent of the actual flow; 95 per cent of the calculated flows lie between errors amounting to +2 and −3.5 per cent; while 82 per cent of flows lie between +2 and −2 per cent of the actual flow. The average error in calculating flows was −0.88 per cent.

ANIMAL EXPERIMENTS DESIGNED TO TEST THE VALIDITY OF THE FUNDAMENTAL PRINCIPLES OF PLETHYSMOKYMOGRAPHY

Does the Plethysmokymograph Measure Blood Flow?—The increase in volume of a limb after inflation of a collecting cuff might be due to trapping of incoming blood within the limb, displacement of fluid and tissue distally from beneath the collecting cuff, or transudation of fluid from the capillaries to the tissues of the limb. In the estimation of blood flow by the plethysmokymographic method it is assumed that the trapping of incoming blood is the chief cause of the increase in volume of the limb. The initial portion of the flow curve represents displacement of fluid and tissue from beneath the collecting cuff and is disregarded in estimating blood flow, as was discussed by Hewlett and Van Zwaluwenburg,[16] or is eliminated from the flow curve mechanically, as discussed by Wright and Phelps,[17] or manually, as discussed by Hewlett and Van Zwaluwenburg.[18] Drury and Jones[19] found that, after occlusion of the return of venous blood from the legs, edema formed in the leg at the rate of 0.017 c.c. per minute per 100 c.c. of tissue when the environmental temperature was 16° C. At 42° C. 0.07 c.c. of edema fluid formed each minute per 100 c.c. of leg tissue. If one applied the latter findings to a human arm of 1,500 c.c. volume even in a hot environment, only about 0.18 c.c. increase in volume would occur during ten seconds after inflation of a collecting cuff. Krogh, Landis, and Turner[20] found that fluid accumulated in tissue spaces when venous pressure exceeded 15 to 20 cm. of water. When venous pressure exceeded 17 cm. of water, the rate of filtration was directly proportional to the increase in venous pressure and an increase in venous pressure of 1 cm. of water increased filtration rate by 0.0023 c.c. per minute per 100 c.c. of arm. For an arm of 1,500 c.c. volume, this filtration rate would yield a maximal increase in arm volume for

ten seconds of about 0.13 cubic centimeter. Lewis[21, 22] found that venous pressure equaled a collecting cuff pressure of 49 mm. of mercury in thirty-nine seconds.

It seemed highly probable from the foregoing investigations that incoming blood could be the only significant factor responsible for the increase in arm volume which occurs during the first ten seconds after a collecting cuff is inflated about the human arm. However, we have been unable to find any direct evidence that such is the case. Hence, the following experiment was performed.

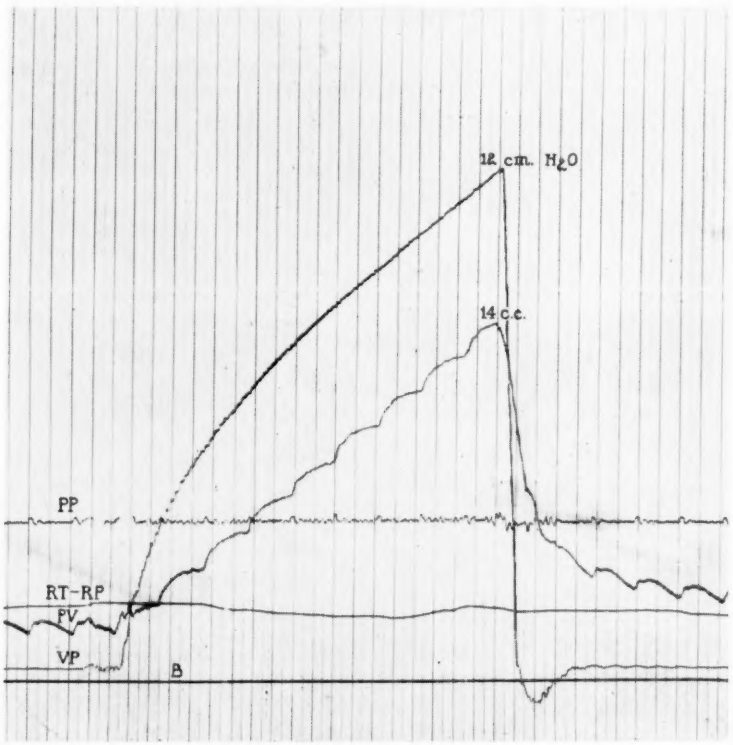

Fig. 10.—Known flows of blood perfused through the isolated hind limbs of a dog by means of a heart-lung preparation. *PP*, Pressure in the plethysmograph recorded by a Marey tambour; *RT-RP*, room temperature and room atmospheric pressure; *PV*, plethysmograph volume recorded by compensating spirometers; *VP*, venous pressure in the hind limbs of the dog; *B*, base line. Heavy vertical lines, time in seconds; light vertical lines, time in one-tenth second.

Using a small dog under pentobarbital sodium anesthesia a heart-lung preparation was made of the upper half of the animal. Then the animal was transected in the midlumbar region. The abdominal aorta and vena cava of the lower half of the animal were cannulated and the entire lower half of the animal was sealed in the plethysmograph with a diaphragm occluding the outlet of the plethysmograph. An inlet for arterial blood and an outlet for venous blood from the lower portion of the animal were passed through the diaphragm and joined to the afore-mentioned cannulae in the abdominal aorta and vena cava. The lower half of the animal enclosed in the plethysmograph was then perfused

with blood by the heart-lung preparation. Occlusion of the venous return was accomplished by clamping the venous outflow tube from the lower half of the animal. Thus flow curves were obtained. A simultaneous record of venous pressure distal to the point of clamping the venous return was made (Fig. 10). Actual flows in cubic centimeters per minute were measured by collecting the blood as it emerged from the venous return tube. Estimation of the blood flow from the flow curve checked closely with the actual flow (Table II). We also noticed that venous pressure began to rise at the instant the venous return tube was clamped (Fig. 10). Furthermore, arterial inflow was unimpeded by a back venous pressure of about 17 cm. of water.

TABLE II. CORRELATION OF ACTUAL BLOOD FLOW THROUGH HIND LIMBS OF DOG AND FLOWS CALCULATED BY THE PLETHYSMOKYMOGRAPHIC METHOD

ACTUAL BLOOD FLOWS C.C. PER MIN. PER 100 C.C.				BLOOD FLOWS CALCULATED FROM FLOW CURVES		
FLOWS COLLECTED BEFORE FLOW CURVES WERE OBTAINED		FLOWS COLLECTED AFTER FLOW CURVES WERE OBTAINED		TRACING	MINIMUM	MAXIMUM
1	62	1	52	A	53.0	62.0
2	60	2	54			
3	58	3	55	B	59.0	62.5
1	46	1	47	A	44.5	44.5
2	48			B	43.0	43.0
1	48.5	1	45	A	46.0	50.0
2	48.5	2	44	B	48.5	48.5
	42		42		45.0	45.0
1	98	1	79		81.0	81.0
2	90					
3	80					
1	82	1	76	A	78.0	78.0
2	82			B	75.0	75.0
1	108	1	104	A	102.5	102.5
2	108			B	102.5	102.5

SUMMARY

A new recording device (compensating spirometer) was developed, analyzed, and compared with other types of recorders commonly used in plethysmokymography.

A new method for calibration of plethysmokymographs is described. Known flows actually collected in cubic centimeters per minute were compared with flows calculated from flow curves. When the compensating spirometer was used for recording blood flow, the discrepancy between actual and calculated values was small enough to permit the conclusion that this new device eliminates most of the errors inherent in other types of recorders and can be used for measuring blood flow in human extremities.

REFERENCES

1. Brodie, T. G., and Russell, A. E.: On the Determination of the Rate of Blood-Flow Through an Organ, J. Physiol. (Proc.) 32: XLVII, 1905.
2. Pezzali, G.: Clinical Significance of Plethysmography, Riforma med. 37: 242, 1921.

3. Herrick, J. F., Essex, H. E., and Baldes, E. J.: Observations on the Flow of Blood of the Kidney, Am. J. Physiol. **99**: 696, 1932.
4. Landowne, M., and Katz, L. N.: A Critique of the Plethysmographic Method of Measuring Blood Flow in the Extremities of Man, Am. Heart J. **23**: 644, 1942.
5. Kubicek, W. G., Sedgwick, F. P., and Visscher, M. B.: Adaptation of Glass Spoon Manometer to Physiological Studies, Rev. Scient. Instruments **12**: 101, 1941.
6. Brodie, T. G.: On Recording Variations in Volume by Air-Transmission. A New Form of Volume-Recorder, J. Physiol. **27**: 473, 1902.
7. Abramson, D. I., Zazeela, Herman, and Marrus, Joseph: Plethysmographic Studies of Peripheral Blood Flow in Man. I. Criteria for Obtaining Accurate Plethysmographic Data, Am. Heart J. **17**: 194, 1939.
8. Abramson, D. I., Zazeela, Herman, and Marrus, Joseph: Plethysmographic Studies of Peripheral Blood Flow in Man. II. Physiologic Factors Affecting Resting Blood Flow in the Extremities, Am. Heart J. **17**: 206, 1939.
9. Ellis, F. W.: Plethysmographic and Vaso-Motor Experiments With Frogs, J. Physiol. **6**: 437, 1885.
10. Turner, R. H.: Studies in the Physiology of Blood Vessels in Man. Apparatus and Methods; a Sensitive Plethysmosphygmograph for a Portion of the Finger, J. Clin. Investigation **16**: 777, 1937.
11. Hewlett, A. W., and Van Zwaluwenburg, J. G.: Method for Estimating the Blood Flow in the Arm; Preliminary Report, Arch. Int. Med. **3**: 254, 1909.
12. Stead, E. A., Jr., and Kunkel, P.: Plethysmographic Method for Quantitative Measurement of Blood Flow in Foot, J. Clin. Investigation **17**: 711, 1938.
13. Prinzmetal, Myron, and Wilson, Clifford: The Nature of the Peripheral Resistance in Arterial Hypertension With Special Reference to the Vasomotor System, J. Clin. Investigation **15**: 63, 1936.
14. Lewis, Thomas, and Grant, Ronald: Observations Upon Reactive Hyperaemia in Man, Heart **12**: 73, 1925.
15. Killian, J. A., and Oclassen, C. A.: Comparative Effects of Water Baths and Mustard Baths at Varying Temperatures on Rate of Peripheral Blood Flow in Man, Am. Heart J. **15**: 425, 1938.
16. Hewlett, A. W., and Van Zwaluwenburg, J. G.: The Rate of Blood Flow in the Arm, Heart **1**: 87, 1909-1910.
17. Wright, G. W., and Phelps, Kenton: A Comparison of Procedures for Increasing Blood Flow to Limbs Using an Improved Optical Plethysmograph, J. Clin. Investigation **19**: 273, 1940.
18. Hewlett, A. W., and Van Zwaluwenburg, J. G.: The Pulse Flow in the Brachial Artery. I. Technic and General Considerations, Arch. Int. Med. **12**: 1, 1913.
19. Drury, A. N., and Jones, N. W.: Observations Upon Rate at Which Œdema Forms When Veins of Human Limb Are Congested, Heart **14**: 55, 1927.
20. Krogh, A., Landis, E. M., and Turner, A. H.: The Movement of Fluid Through the Human Capillary Wall in Relation to Venous Pressure and to the Colloid Osmotic Pressure of the Blood, J. Clin. Investigation **11**: 63, 1932.
21. Lewis, T.: Force Exerted by Minute Vessels of the Skin in Contracting, Heart **11**: 109, 1924.
22. Lewis, Thomas: The Blood Vessels of the Human Skin and Their Responses, London, 1927, Shaw & Sons, Ltd.

DEVICES FOR RAPID RECORDING OF MULTIPLE AND SPECIAL ELECTROCARDIOGRAPHIC LEADS

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PERHAPS the greatest advance in modern electrocardiography is the employment of special exploring leads (direct and semidirect) that are physically closer to a particular part of the heart and register more intimately its electrical activity. The theoretic importance of such leads was long recognized by workers in this field, but for the sake of simplicity and convenience, so that electrocardiography could come into widespread use, the limb leads were emphasized as the exclusive standard. Now with the anterior surface of the heart being studied by multiple chest leads, the posterior surface and base by multiple esophageal leads, and the individual, separate chambers of the heart (such as the right auricle) by leads specially suited to the study, promise for real advance in electrocardiography is high. It is important, however, to determine what the particular usefulness of a special lead is and also which leads are so informative that they should by right be used routinely in electrocardiography; the added information will more than compensate for the extra effort in recording the tracings. Definite headway has been made in this regard, but much menial work such as obtaining normal standards for a given lead, comparing it with similar ones, and outlining its limits of usefulness still remains to be done. It was felt that this task could be expedited and made less unpleasant if special apparatus were designed to accomplish this type of multiple lead recording with speed. The apparatus to be described* is flexible enough to record speedily almost every lead that has been proposed to date, and should be useful not only in this preliminary period when the standard of special multiple leads is being established, but also later in the routine recording of the multiple lead electrocardiogram which promises to be the tracing of the future.

Switch Box.—The switching device which permits rapid recordiag of multiple leads without the moving of wires on the patient or the machine is shown in Fig. 1. It consists of a main selector switch and two auxiliary switches which are interposed between the patient and the electrocardiograph. All manipulations for the selection of leads are accomplished at this switch, the electrocardiograph always recording the selected lead on its fixed Lead II setting. Five shielded wires run from the patient to the switch, one from each extremity (the right leg being used as a ground when the other three extremities are connected to the Wilson central terminal) and a fifth one from either the precordium or any other area to be explored. From the switch run the RA, LA, and LL wires of the electrocardiograph at connector marked ECG. Although only Lead II (RA and LL) is necessary for the recording, the switch makes all its grounding connections for any selected lead through its LA terminal. This insures a base line free of A.C. and other extraneous interferences, so that the switch box may be used with the newer types of direct-writing electrocardiographs that are so sensitive to A.C. interference.

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*Made by Medical Designs, Brooklyn, N. Y.

The main selector switch permits recording not only of the conventional leads, but also of the chest leads most commonly employed: precordium-right arm (CR), precordium-left arm (CL), precordium-left leg (CF), and precordium-Wilson central terminal (V, in which all three extremities are connected through equal 5,000 ohm noninductive resistances to furnish a point of zero potential).[1] There are also settings on the switch for obtaining the potentials of the right arm, left arm, or left leg, that is the unipolar limb leads, with the Wilson terminal as the indifferent electrode (V_R, V_L, and V_F). The complexes obtained with the V_R, V_L, and V_F leads tend to be small. Tracings of higher amplitude are secured by shunting out of the

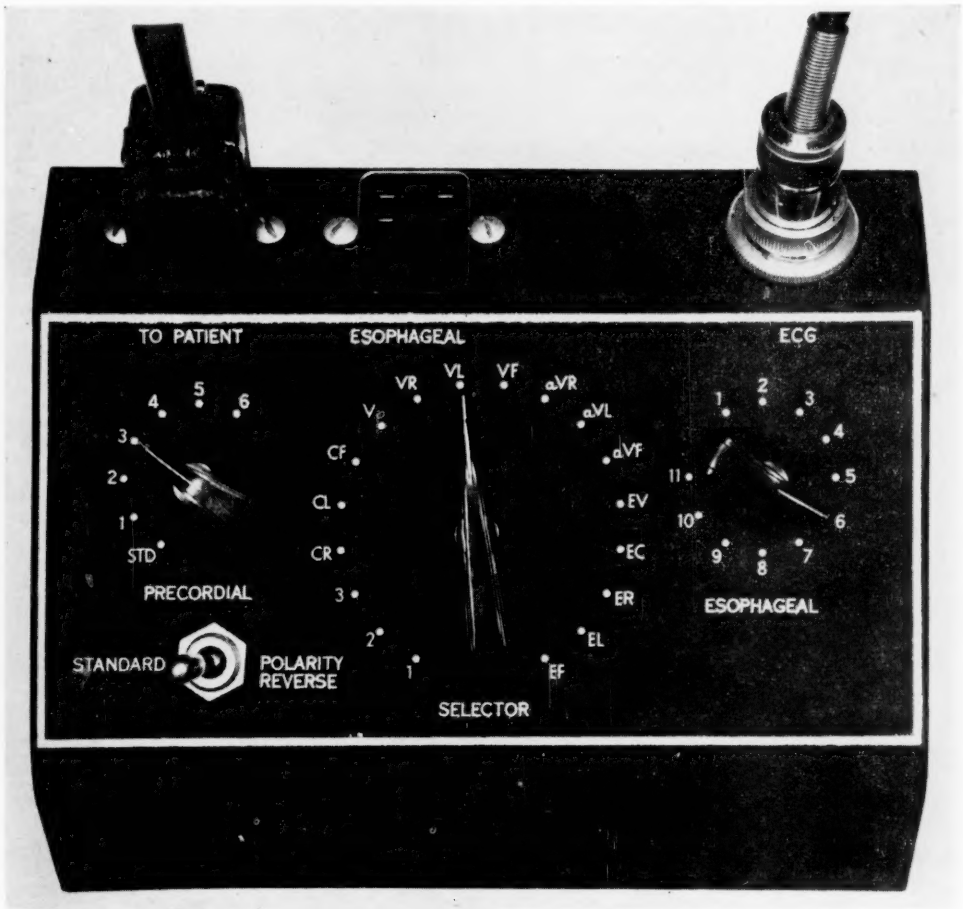

Fig. 1.—Switch box.

circuit the 5,000 ohms resistance from the extremity that is being explored while keeping the resistors of the other two extremities in the Wilson hookup. These, the augmented Wilson unipolar leads, can be recorded by turning the switch to the aV_R, aV_L, or aV_F settings.

As seen in Fig. 1, there are two adjuvant switches used in conjunction with the main selector switch; the left-hand one is for multiple leads taken from the front of the heart (precordial), and the right-hand, from the back of the heart (esophageal). For taking single precordial leads the exploring electrode (fifth patient wire) is used, and the precordial switch is set at precordial (STD). If the multiple precordial lead belt, to be described later, is used, the tracings from the six precordial electrodes are taken in succession by use of switch settings

1 to 6 inclusive. The main switch permits a choice of four indifferent points for precordial registration, that is, the right arm (CR), the left arm (CL), the left leg (CF), and the Wilson terminal (V). For esophageal tracings the eleven-terminal esophageal lead, to be described later, is plugged into the selector switch and tracings from each of the eleven esophageal levels are taken in succession on the appropriate setting of the esophageal switch. Here there is a choice of five indifferent points for coupling with the esophageal lead: right arm (E_R), left arm (E_L), left leg (E_F), precordium (E_C), or Wilson terminal (E_V). For the past thirteen years we[2] have been accustomed to recording the esophageal lead with the exploring electrode connected to the RA terminal and the indifferent electrode to the LL terminal, so that the resultant tracing (taken the same way as Lead II) should resemble the

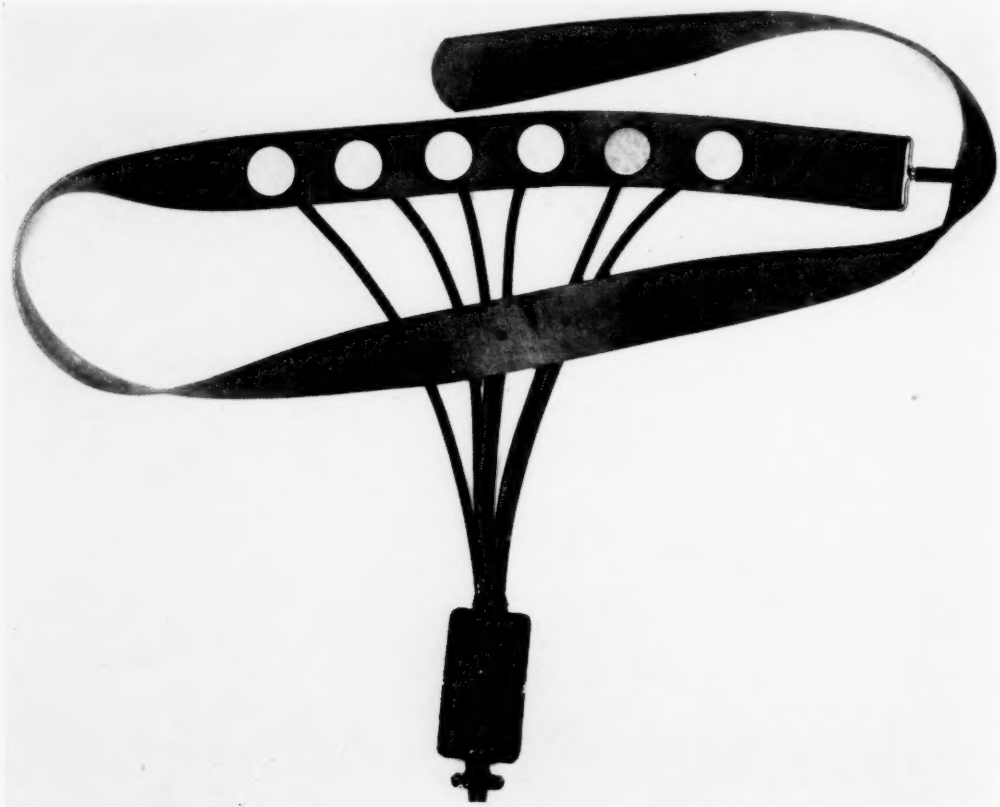

Fig. 2.—Multiple precordial lead belt.

precordial and conventional leads. This direction was followed by Brown[3] but is opposite to that used by Nyboer,[4] Graybiel and White,[5] and others. It is a simple matter to reverse the direction of the tracing by use of a simple throw switch marked Polarity Reverse on the left side of the switch box. This switch reverses the polarity not only of the esophageal lead, but also of any of the other leads when desired.

Multiple Precordial Belt.—A modification of the elastic rubber belt described by Geiger and Goerner[6] has been found very effective in the rapid recording of multiple precordial leads (Fig. 2). The belt is made of high-quality flat rubber belting about 1 inch wide and 42 inches long. Six flat German silver disk electrodes about 3 cm. in diameter are spaced 4 cm. apart toward one end of the belt. Soldered wires run from the disks through a six-prong Jones connector and join the other five leads to form the eleven-lead patient's cable (Fig. 3).

This cable plugs into the switch box at the connector marked To Patient. A hook on the free end of the strap fits into the proper hole punched in the belt, making for easy adjustment of the elastic belt to the size of the chest. The only precordial placements that need be determined are the positions of electrodes 1 (right edge of sternum in the fourth interspace) and 6 (midmaxillary line in the fifth interspace). The average technician locates these two points

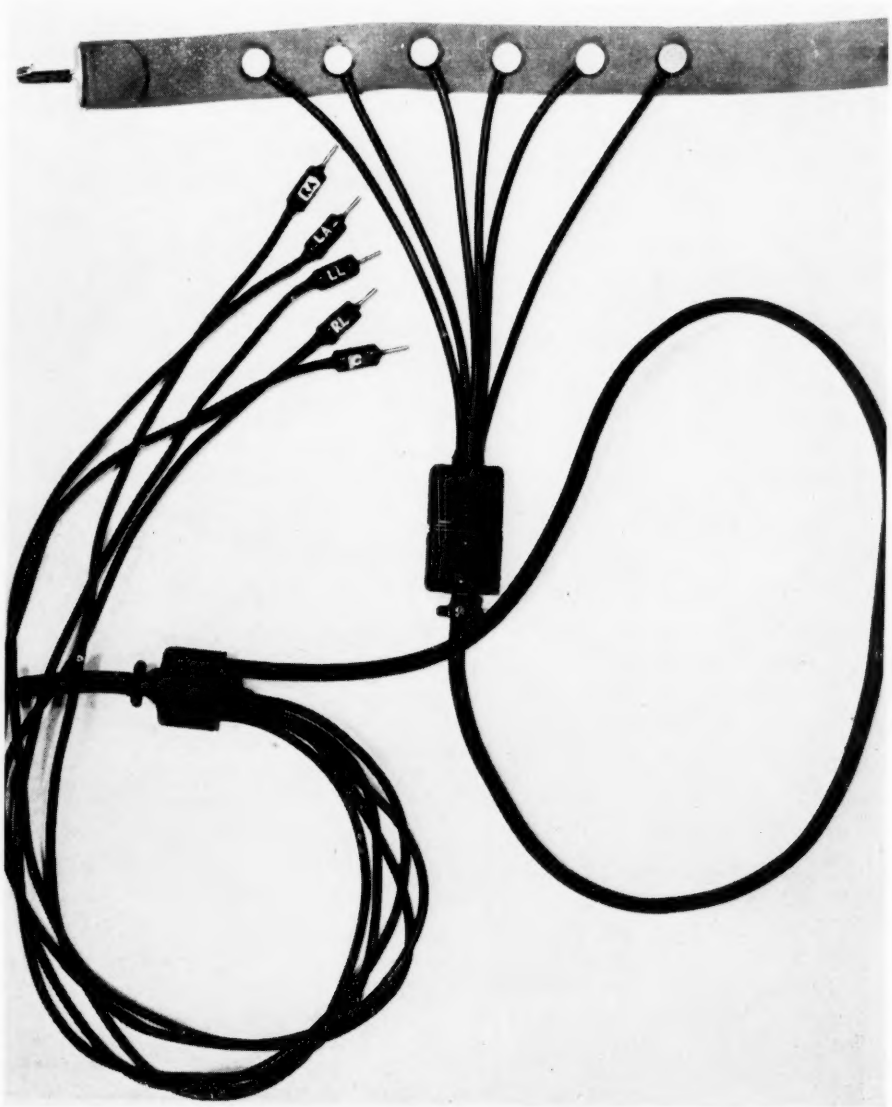

Fig. 3.—Eleven-lead patient's cable.

easily enough. The other four electrodes lie automatically at equal distances from 1 to 6. The saving in time with this technique is appreciable. For still greater speed, the following practical points may be noted. The rubber strap is laid across the arms of the electrocardiographic chair before the patient seats himself, avoiding the clumsy procedure of passing the belt around the patient's back. A thin layer of electrode paste is applied to

the six electrodes. The sixth electrode is first placed in the proper position. With the free end of the belt held in the left hand, the belt is put under tension with the right hand until electrode 1 has reached the proper position (right sternal edge). It is only then that the electrodes 1 to 5 are permitted to touch the chest. One can thus see the fifth disk falling into its proper position, then the fourth, the third, and so forth. If the amplifier type of electrocardiograph is used, no further rubbing of the skin is necessary. With the string type, the electrodes may have to be rubbed up and down to reduce skin resistance, particularly if the chest is hairy. The size of the female breast introduces no difficulty with this technique, for the breasts are always lifted and the belt is set in position beneath the mammary folds.

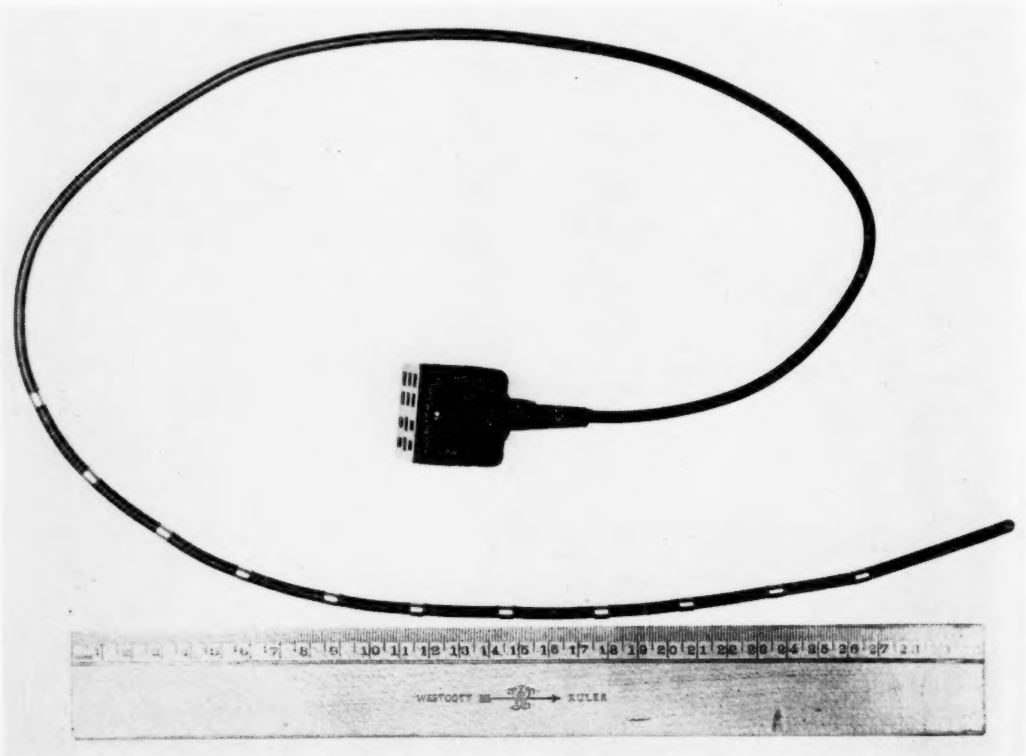

Fig. 4.—Eleven-electrode esophageal lead.

Esophageal Lead.—The esophageal lead designed for this study (Fig. 4) is similar to Nyboer's.[4] The essential difference is that by an improvement in production technique an eleven-electrode lead can be built into a No. 12 Fr Levin tube, whereas to date this could be accomplished only in the larger 14 Fr or 16 Fr size. The 12 Fr tube can be readily passed, even in children. A special Levin tube which has no openings in the end is used so that no secretions can penetrate to the inside. Eleven narrow German silver rings spaced 3 cm. apart are attached to the tube. A separate insulated wire leads from each ring through the tube to its appropriate post in a twelve-prong Jones connector plug. The twelfth prong of the plug connects to the electrical shielding of the cable that runs to the switch box (not shown in Fig. 4). The technique in use with the esophageal lead is as follows: The patient is first reassured about the safety of the procedure. The rubber tip of the esophageal lead is lubricated for about an inch or two with a little mineral oil. Through the more patent

nostril the tube is rapidly passed beyond the posterior pharynx, which is likely to be the most sensitive part, down into the esophagus. The patient is asked to sip some water through a straw or drinking tube while the lead is being passed. This preoccupation with drinking permits the lead to be passed within about ten seconds to the proper level (the lowest electrode being placed 55 to 60 cm. from the nares, as indicated by a mark on the tube). It is rarely necessary to use a local anesthetic for nose or pharynx. The patient is instructed to take a deep breath if uncomfortable or nauseated. In general, within a minute the patient will have become calm after the initial excitement of passing the tube. It will take, however, another five minutes or so for the esophagus to make its adjustment to the presence of the Levin tube in its lumen. During this time the position of the esophageal electrodes may be checked fluoroscopically if desired, and the other connections can be made from the switch box to the patient's extremities and precordium. By the time the conventional leads and precordial leads have been recorded, the esophageal lead can be taken from each of the eleven esophageal levels in succession, using the esophageal switch. This five- or ten-minute waiting period after passing the esophageal lead, as well as the electrical shielding of the esophageal cable, cuts down the drifting of the base line that is such a disturbing feature of esophageal electrocardiograms. It is to be noted that with the eleven electrodes spaced 3 cm. apart the entire base and the posterior aspect of the heart are explored as far as the esophagus permits without the necessity of moving the esophageal lead up or down from its original placement. In contrast, most esophageal leads used until now have employed a single or only a few electrodes and have had to be manipulated up and down the esophagus to cover the electrical field desired. The consequent retching and esophageal contractions cause much drifting of the base line and numerous artifacts in the tracings. The amplifier type of machine is better suited in general for the recording of esophageal electrocardiograms because of the high resistances found in the esophagus. On the other hand, we have taken very satisfactory tracings using the eleven-electrode esophageal lead and a standard string galvanometer.

COMMENT

The devices outlined are being utilized in an extensive study of the usefulness of the many special and multiple leads suggested for the more intimate exploration of the heart and its separate chambers. Our findings will be reported at a later date when our data are more complete. It is considered worth while, however, to describe at this time the different devices designed to speed up the investigation, for similar studies must be made by numerous investigators and the findings correlated before a proper evaluation can be made of the usefulness of any proposed lead. The end in view of such studies is the establishment of the value of certain leads to the point where their inclusion in routine electrocardiography would be justified. There are other leads which, although not of such universal significance as to become routine, may still be found most suitable for a special study of a particular part of the heart. Thus the exact usefulness and also the limitations of a derivation such as the esophageal lead, coming in contact with the left auricle and the base of the left ventricle, could be established. This type of study is tedious and time consuming; any device that simplifies the procedure appears to us worthy of description.

The main selector switch with its adjuvant multiple precordial and esophageal switches permits the rapid recording of practically all leads which have been proposed to date. The multiple esophageal and precordial leads can be connected further with a number of points as the indifferent electrode (right arm, left arm, left leg, Wilson central terminal, etc.), and the value of these different derivations can be compared. Although from the theoretic point of

view it may be best to couple all exploring leads with the Wilson terminal, empirically we, as well as others, have found instances in which other derivations were more informative. Whichever derivations will stand the test of time, it is likely that they can be easily registered with the multiple switch box described. Great pains have been taken to shield all the leads used in this study so that the devices could be employed with any electrocardiograph, even the direct-writing electrocardiographs that are so sensitive to A.C. interference. The machines that incorporate the instomatic principle of bringing the beam to the mid-line before switching to the next lead are particularly suitable to the rapid recording of multiple leads. Here one need only press the instomatic button and turn the multiple lead switch from one position to the other, and the machine is ready within a few seconds to record the new selection.

The technique described for taking multiple precordial leads is similar to the one used by Geiger and Goerner[6] and is advocated because of its simplicity and speed. Our experience with this belt technique almost exactly duplicates theirs. The difference between records obtained with this technique and the individual placements advocated by the American Heart Association[7] appear minimal and of little, if any, clinical significance. Results can be duplicated better with the belt than with the six individual placements, whether the physician or the technician determines them. Furthermore, in the belt technique, the fixed relation of the electrodes to the thorax rather than to the heart appears more sound, theoretically, in the study of many aspects of heart disease (axis deviation, bundle branch block, cardiac hypertrophy) than does the standard method.

The esophageal lead which we introduced in 1934[2] has not yet been widely employed, mainly because of technical difficulties. These have been overcome largely by the new design of the tube which can be passed easily enough to be used when indicated in any case except perhaps in terminal decompensation or during the first few weeks of a coronary occlusion. The switch which permits rapid recording from each of the eleven esophageal levels in succession saves so much time that the taking of an esophageal lead no longer can be looked upon as a formidable procedure. Once the physician convinces himself by actual trial that this is true, he can readily transmit this confidence to the patient, and the field of usefulness of the esophageal lead widens immeasurably.

SUMMARY AND CONCLUSIONS

Three devices designed for the rapid registration of multiple leads are described. The multiple switch box with a limb lead selector, multiple precordial lead selector, and multiple esophageal lead selector is the main device. Aside from the different chest leads (CR, CL, CF, and V), Wilson's unipolar limb leads and augmented unipolar limb leads can be obtained readily. A single precordial lead or six multiple precordial leads can be coupled with any of four indifferent points, and the eleven-electrode esophageal lead with any of five indifferent points.

An elastic multiple precordial lead belt is described which facilitates the rapid recording from six precordial areas by plugging into the switch box.

A thin eleven-electrode esophageal lead which plugs into the selector switch is described. The procedure of recording an esophageal lead has been simplified so that it may be applied to any case except serious acute cardiac incidents.

It is hoped that these devices may prove of use to other investigators who are studying the comparative usefulness of special and multiple leads in electrocardiography.

We are deeply indebted to the late Dr. Frank Liberson and Dr. I. W. Held whose constant inspiration made this study possible.

REFERENCES

1. Wilson, F. N., Johnston, F. D., Macleod, A. G., and Barker, P. S.: Electrocardiograms That Represent the Potential Variations of a Single Electrode, Am. Heart J. 9: 447, 1934.
2. Lieberson, A., and Liberson, F.: An Internal Electrocardiograph Lead, Proc. Soc. Exper. Biol. & Med. 31: 441, 1934.
3. Brown, W. H.: A Study of the Esophageal Lead in Clinical Electrocardiography, Am. Heart J. 12: 1, 1936.
4. Nyboer, J.: The Normal and Abnormal Esophageal Electrocardiogram With Particular Reference to Myocardial Infarction, Am. Heart J. 22: 469, 1941.
5. Graybiel, A., and White, P. D.: Electrocardiography in Practice, ed. 2, Philadelphia, 1946, W. B. Saunders Company, p. 444.
6. Geiger, A. J., and Goerner, J. R.: A Simplified and More Standardized Technique for Recording Multiple Precordial Electrocardiograms, Am. Heart J. 32: 163, 1946.
7. a. Standardization of Precordial Leads; Joint Recommendations of the American Heart Association and the Cardiac Society of Great Britain and Ireland, Am. Heart J. 15: 107, 1938.
   b. Supplementary Report, Am. Heart J. 15: 235, 1938.
   c. Second Supplementary Report, Am. Heart J. 25: 535, 1943.

DETERMINATION OF VOLATILE REDUCING SUBSTANCES (ALCOHOL OR ETHER) IN BLOOD AND GASES USING BARIUM DIPHENYLAMINE SULFONATE AS AN INDICATOR FOR CHROMIC ACID TITRATION

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IN THE determination of volatile reducing substances (primarily alcohol and ether) in blood or in air the usual procedure consists of absorbing these volatile organic substances in sulfuric acid, adding a known amount of potassium dichromate, and oxidizing the alcohol or ether to acetic acid by the chromic acid. The excess dichromate is then determined either colorimetrically or by titration.

With the colorimetric method as used by Gibson and Blotner (1938) and Newman and Abramson (1942), the organic substances are oxidized to acetic acid by chromic acid and the dichromate residue, consisting of chromic acid and chromic sulfate, is determined by absorption of light in the region of wave length 440 to 480 millimicrons. This method, while rapid, has the three following disadvantages. (1) The relation between the logarithm of the galvanometer reading and ether (or alcohol) concentration is not a straight line but is slightly convex. (2) Since light is absorbed by both dichromate and chromic sulfate it is necessary to calibrate the apparatus with ether (or alcohol) solutions of known concentration. Due to the highly volatile nature of ether and alcohol and the affinity of alcohol for water, the preparation, storage, and dispensing of standard solutions for precise work is a task requiring unusual precautions in technique. (3) For small quantities of blood the dichromate-chromic sulfate mixture has low spectrophotometric sensitivity.

The titration method has been used widely. Excess dichromate after oxidation is titrated with a suitable reducing solution and a redox indicator. Iodometric titration of excess nonreduced dichromate was used by Shaffer and Ronzoni (1923, Newman (1936), McNally and Coleman (1944), Widmark (1922), and others; while Harger (1935), Cavett (1938), Levine and Bodansky (1939), and Fisk and Nelson (1941) preferred ferrous sulfate solution containing methyl orange. The methyl orange-ferrous sulfate solution was used in preference to the older iodometric titration because of the unsatisfactory titration end point of the older method.

In the present investigation the colorimetric method using the Evelyn colorimeter was used and was rejected for the reasons already given. Titration

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using the ferrous sulfate-methyl orange mixture, was tried but rejected because the titration end point was not sharp. The redox indicator barium diphenylamine sulfonate was next investigated, using the titration procedure described by Kolthoff and Sandell (1938). This indicator, in the presence of phosphoric acid, gave a sharp end point with the color changing from violet-blue to colorless. For titrating a sample of dichromate equivalent to one milligram of ether with 20 mls. of a solution of ferrous sulfate, a fraction of a drop of the reducing solution was sufficient to cause a sudden and striking change of color. This indicator was adopted for the determination of alcohol and ether in blood or in air with the procedure which will be described.

METHODS

Reagents.—Ferrous sulfate, stock solution. Dissolve 50 Gm. of $FeSO_4 \cdot 7H_2O$ in 150 ml. of water. Add 30 ml. of concentrated sulfuric acid and dilute to 250 milliliters. In a stoppered flask the solution will undergo only slight oxidation.

Barium diphenylamine sulfonate, stock solution. Dissolve 0.1 Gm. barium diphenylamine sulfonate in 100 ml. of water.

Ferrous sulfate indicator solution for titration. To 15 ml. of the ferrous sulfate stock solution and 20 ml. of the indicator stock solution add water to 2000 milliliters.

Potassium dichromate, standard solution. Weigh out exactly 5.295 Gm. $K_2Cr_2O_7$ (Mallinckrodt's analytical reagent), dissolve in water and dilute to one liter. One milliliter of this solution is equivalent to 1 mg. ether.

Sulfuric acid, C.P. Concentrated.

Phosphoric acid, C.P. 85 per cent.

Procedure for Determining Ether in Blood.—Widmark flasks (Cavett, 1938) of 50 ml. capacity with T-24 standard tapered Pyrex stoppers were made, Fig. 1, *A*. It is desirable in preparing these flasks to make the glass rod by means of which the cup is suspended from the stopper as short as possible. This prevents the cup (2.5 ml. capacity) from dipping into the chromic acid at the bottom of the flask.

To determine ether in blood, the blood is drawn into a syringe with a drop of saturated potassium oxalate filling the needle and needle tip of the syringe. The blood is drawn with care to exclude bubbles in the syringe and is discharged into a two-way stopcock pipette which contains approximately 0.25 ml. of water in one arm, Fig. 1, *A*. This pipette is made to contain approximately 1 ml. in the lower arm. The volume of the lower arm is carefully determined with mercury or standard solution. Blood is discharged from the syringe as shown in Fig. 1, *A*, and it ascends in the lower arm through the stopcock to the upper arm which does not contain the water. Approximately 0.5 ml. of blood will ascend above the stopcock. The blood exposed to air as it ascends in the pipette will have lost ether to the air above its surface. This blood will be rejected. The stopcock is now closed, the syringe removed from the pipette, and the lower

tip of the pipette held above the cup of the Widmark flask. The stopcock is turned to the arm containing the water, the blood is drained into the Widmark cup and is washed from the pipette with the 1 ml. of water. The Widmark flask is stoppered immediately. A syringe pipette also has been used and found to be satisfactory.

Either 1.0 or 1.5 ml. of standard dichromate solution from a dispensing burette are placed in the bottom of the Widmark flask and 3.0 ml. of concentrated sulfuric acid are added. Similar quantities of dichromate and sulfuric acid are measured out for a blank and placed in a glass-stoppered 50 ml. Erlenmeyer flask. The blood and wash water are placed in the Widmark cup as

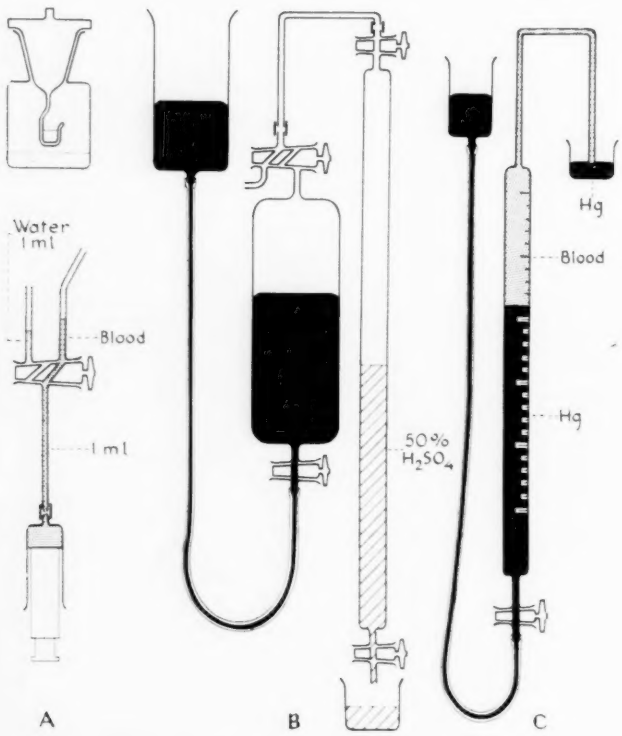

Fig. 1.—*A*, Blood pipette and syringe, Widmark flask; *B*, gas sampling bulb and ether absorber, *C*, burette for storage and dispensing of solutions with volatile solutes.

described and both the Widmark flask and the Erlenmeyer flask transferred to an oven at 80° C. for four hours. The solutions are removed then and it will be observed that the blood has hardened to a cake by evaporation. After cooling, 3 ml. of concentrated phosphoric acid are added and the solutions are titrated in the same flasks with ferrous sulfate containing diphenylamine sulfonate. The number of milligrams of ether in 100 ml. of blood is given by the formula:

$$\text{Ether (mg.) in 100 ml. of blood} = \frac{T_B - T_X}{T_B - 3\ e} \cdot \frac{100}{V} \cdot N$$

where T_B is the titration value of the blank (milliliters ferrous sulfate solution); T_X, the titration value of the unknown; V, the volume of the sample to be analyzed; N, the number of milliliters of the standard dichromate solution; and e, the reducing equivalent in milliliters of ferrous sulfate solution of 1 ml. of concentrated sulfuric acid which is determined as explained later.

Procedure for Determining Ether in Air.—The air-ether mixture is collected in a gas sampling bulb of approximately 60 ml. whose exact volume is determined by calibration. The sampling bulb has a one-way stopcock at the bottom and a two-way stopcock at the top, and is connected to the bulb as shown in Fig. 1, *B*. The upper stopcock of the gas sampling bulb is joined with a short rubber connection to a glass capillary tube which is then connected to a long 100 ml. tube (an old 100 ml. burette can be used for the purpose). The 100 ml. tube has a stopcock at the top and bottom, and at the start is filled by suction to the upper stopcock with 50 per cent sulfuric acid. The gas from the sampling bulb is transferred to the long absorber tube and displaces the sulfuric acid. After all the gas has been transferred, approximately 10 ml. of ether-free air are washed from the stopcock at the top of the sampling bulb through the connecting tube to the absorber. The stopcocks at the bottom and top of the absorber are closed, the connecting tube is disconnected from the sampling bulb, and the absorber is shaken for about one minute and allowed to stand ten minutes. The contents of the absorber are then transferred to a 50 ml. volumetric flask, the absorber is washed with approximately 5 ml. of 50 per cent sulfuric acid which is transferred to the volumetric flask, and the flask is made up to 50 ml. with 50 per cent sulfuric acid. Three milliliters of the contents of the volumetric flask are placed in a glass-stoppered Erlenmeyer flask containing 1.50 ml. of the standard dichromate solution; the flask is allowed to stand at room temperature for thirty minutes. This solution and a flask containing 3 ml. of 50 per cent sulfuric acid and 1.00 ml. of standard dichromate are titrated at the same time. The amount of ether in 100 ml. of the air-ether mixture is computed from the following formula:

$$\text{Ether (mg.) in 100 ml. of air-ether mixture} = \frac{T_B - T_X}{T_B - 1.5\ e} \cdot \frac{100}{V} \cdot \frac{50}{3} \cdot N$$

where T_B is the titration value of the blank in the flask; T_X, the titration value of the unknown; e, the reducing equivalent of the sulfuric acid used; V, the volume of the gas sampling tube in milliliters; N, the number of milliliters of dichromate; the volume of the volumetric flask, 50 ml.; and the pipette volume, 3 milliliters.

Test for Reducing Power of Sulfuric Acid.—Sulfuric acid contains a variable amount of reducing material, possibly SO_2, which reduces ferrous sulfate. In many samples of sulfuric acid there are no reducing substances. For accurate analyses an estimate must be made of the reducing power, e, of the sulfuric acid used in the procedure. This is done by placing in a series of six Erlenmeyer flasks 1 ml. of the standard dichromate and 0, 1, 2, 3, 4, and 5 ml. of

concentrated sulfuric acid. To each flask, 3 ml. of concentrated phosphoric acid are added and the solutions titrated. The titration values are plotted against milliliters of sulfuric acid, and the reducing power, e, per milliliter of sulfuric acid determined. This titration must be made for each new bottle of sulfuric acid used. The value of e has not exceeded 0.5 ml. of the ferrous sulfate solution used for titration. All samples of phosphoric acid tested were found to be free of reducing substances.

Test for Oxidation of Acetic Acid.—In the Nicloux oxidation of ether and alcohol, as given by the following equations, two assumptions are made, namely (1) that all of the alcohol (or ether) is oxidized to acetic acid, and (2) that none of the acetic acid is oxidized.

$$4K_2Cr_2O_7 + 3(CH_3CH_2)_2O + 16H_2SO_4 \text{ —— } 4K_2SO_4 + 4Cr_2(SO_4)_3 + 6CH_3COOH + 19H_2O$$

$$2K_2Cr_2O_7 + 3CH_3CH_2OH + 8H_2SO_4 \text{ —— } 2K_2SO_4 + 2Cr_2(SO_4)_3 + 3CH_3COOH + 11H_2O$$

If these assumptions are valid, the oxidation proceeds as shown by the equations and an equivalence can be computed whereby 1 milligram of ether is equivalent to 5.295 mg. of potassium dichromate and 1 mg. of alcohol is equivalent to 4.259 mg. of potassium dichromate. It is necessary to determine, as pointed out by Shaffer and Ronzoni but ignored by many later workers, whether or not oxidation is complete and whether or not acetic acid is oxidized. A number of experiments were performed to determine whether acetic acid was oxidized under the conditions of the experiment. A solution of acetic acid containing 1.68 mg. of acetic acid per milliliter of solution was prepared and tested for oxidation. Four solutions, each containing 1 ml. of standard dichromate solution and 3 ml. of concentrated sulfuric acid, were prepared (in quadruplicate) and treated in the following way: (A) Blank—titrated with ferrous sulfate—diphenylamine sulfonate solution at room temperature; (B) 1 ml. of acetic acid solution added and titrated at room temperature; (C) Blank (chromic acid alone), heated for four hours at 80° C.; (D) 1 ml. of acetic acid solution added and heated in an oven for four hours at 80° C. The results are contained in the following table:

SOLUTION	CONDITION	TITRATION VALUE	EQUIVALENT ETHER (MG.)
A Blank	room temperature	23.80	1.00
B Blank with 1 ml. of acetic acid	room temperature	23.83	1.00
C Blank	80° C. for 4 hr.	23.09	1.00
D Blank with 1 ml. of acetic acid	80° C. for 4 hr.	22.99	1.009

It is to be noted that the oxidation of acetic acid equivalent to 1 mg. of ether is less than 1 per cent in terms of the error involved in ether analysis, and hence is so small that it can be neglected. However, on comparing A and C it is to be noted that there is a loss of approximately 3 per cent in the oxidizing power of chromic acid when kept for four hours at 80° C. This result requires that the blank chromic acid be treated in the same manner as the chromic acid which absorbs ether, namely being kept with the ether absorbing solution at 80° C. for four hours.

Preparation of Standard Solutions of Ether and Alcohol.—The method, when adequately tested, does not require standard solutions of ether (or alcohol) for calibration since the sole standard is the potassium dichromate solution. For checking the method, however, standard solutions are necessary and the preparation of these involves certain difficulties. Solutions of ether are difficult to prepare due to the high volatility of ether. Alcohol solutions are difficult to prepare due to their volatility and tendency to absorb water from the air.

The best method for storing and dispensing the solutions was found to be a burette constructed as shown in Fig. 1, *C*. Three burettes were constructed: one for a pure ether solution made from a 5 ml. Mohr pipette; one for blood made from a 10 ml. Mohr pipette; and a 25 ml. burette for an ether stock solution containing 10 mg. of ether per milliliter. Pure ether was dispensed from the ether burette and the weight computed from density tables. A stock solution of 100 ml. of ether containing 10 mg. per milliliter was made by pipetting 1.43 ml. of ether at 24° C. into 90 ml. of water in a volumetric flask. The solution was rapidly diluted to volume, stoppered, shaken, and allowed to stand overnight. Twenty-five milliliters of this solution were drawn from the bottom of the flask and transferred to the burette for a standard solution; the remainder was discarded. A solution of ether in blood was made by adding 10 ml. of the standard solution to 90 ml. of blood and by withdrawing 10 ml. of this solution from the bottom of the volumetric flask into the blood burette. This procedure was adopted after many trials of other less satisfactory gravimetric methods.

Test for Recovery From Prepared Solutions of Ether in Blood.—A recovery test from prepared solutions whose ether concentrations are known is a test for determination of completeness of transfer of ether and completeness of oxidation. At a temperature of 80° C. it was found that all of the ether distilled from 1 ml. of a solution of ether in water in two hours. Distillation from blood was slower and required four hours. Oxidation of ether in chromic acid prepared as described is complete in thirty minutes at room temperature. Solutions of ether in blood were prepared and the recovery of ether measured. The results were as follows for a solution made to contain 100 mg. ether per 100 milliliters: solution A, 105.0 mg. per 100 ml.; solution B, 104.0 mg. per 100 milliliters.

Organic volatile matter in 100 ml. blood was 2.5 milligrams.

Corrected value for ether in blood was 102.0 mg. per 100 milliliters. An error of 2 per cent was involved which was probably due to the errors involved in preparation of the standard solutions.

Distillation Time.—A test was made to determine the time required for distillation in the oven at 80° C. This time will vary with the surface area and volume of the two solutions in the Widmark flask; that is, the ether-containing solution in the cup and the receiving solution (chromic acid) at the bottom of the flask. Following are the data on recovery of ether from a standard solution of ether in blood.

2 hours in oven	90.2 per cent
3 hours in oven	95.5 per cent
4 hours in oven	99.2 per cent

With the Widmark flasks used, four hours in the oven were required for complete distillation although distillation was 90 per cent complete after two hours. Distillation is not complete until the blood sample is hard and caked.

DISCUSSION

The Widmark method of determining ether (or alcohol) in blood has the advantage of eliminating a distillation process, which permits a considerable saving of time. It does require, however, a wait of four hours for ether to diffuse from the solution in the cup to the receiving solution at the bottom of the flask. Where speed in analysis is not essential, the convenience of this method is an advantage. The four-hour period in the oven will cause a loss of the oxidizing power of the dichromate which will amount to as much as 5 per cent. This can be corrected for by placing the blank chromic acid in the oven with the ether sample. The method used by Levine and Bodansky (1940), in which the sample of blood containing alcohol was absorbed by a filter paper and suspended within a flask containing chromic acid, was found to be unsatisfactory due to the dropping of invisible particles of dust or dry material from the paper into the chromic acid (Anderson, 1942). Rapid transfer is effected by this method but irregular results are obtained. In using chromic acid for absorption of less than milligram quantities of ether, it is necessary to use scrupulously clean glassware and to avoid dust particles. All open flasks must be covered and only the cleanest of stirring rods, kept dust free, can be used. It was the customary practice in a series of titrations to discard the first blank titration which was usually low, probably due to dust. If rapid analysis is necessary and if the delay due to ether transfer in the Widmark flasks is to be avoided, a distillation similar to that described by McNally and Coleman (1944) could be used.

A precaution which is recommended is that chromic acid cleaning solution be avoided in cleaning the glassware. Trisodium phosphate cleaning solution has been found to be satisfactory for this purpose.

The reagents to be dispensed, including ferrous sulfate, sulfuric acid, phosphoric acid, and standard dichromate, are all delivered from burettes. A 2 ml. burette is used for the standard dichromate, and 50 ml. burettes are used for the concentrated acids. These acids are delivered to the burettes from an all-glass reservoir and connecting system by pressure. Phosphoric acid is used as a stopcock lubricant for the burettes containing the strong acids, but is not highly satisfactory since a slight sticking of the stopcocks will occur. Some of the silicone greases might be useful for this purpose. A minimum amount of grease must be used on the stopcocks of the burettes containing the potassium dichromate and the ferrous sulfate.

In using the redox indicator, barium diphenylamine sulfonate, the indicator can be added in a constant amount to the dichromate solution. The indicator itself undergoes oxidation and reduction, hence a correction is theoretically required. This correction cancels out and the step in the analysis which requires addition of the indicator can be eliminated if the indicator is added to the

ferrous sulfate reducing solution. The total reductant then includes both the indicator and the ferrous sulfate, and the mixture is standardized against a known amount of potassium dichromate. This procedure has the disadvantage that when only a small amount of chromic acid remains unreduced in the solution and only a few milliliters of reducing solution are needed, the amount of indicator present may be insufficient to produce a well-defined color. This difficulty can be avoided by adding sufficient excess dichromate, which is always a safe procedure in any event since if the amount of dichromate is insufficient for the ether present the analysis is spoiled. An estimated excess of 0.2 to 0.5 ml. of standard dichromate which required 4 to 10 ml. of the ferrous sulfate indicator solution was found to be satisfactory.

In evaluating the present method in comparison with other methods described in the literature (Levine and Bodansky, 1939), the present method is found to be unique in possessing a sharp, well-defined, and highly chromogenic end point. The titration method relies for standardization and calibration on a single standard solution of potassium dichromate, which is a reagent readily available in a highly purified form and therefore makes an excellent standard. It is not necessary, as is required with the colorimeter method, to calibrate with standard solutions of alcohol or ether. These are required in the colorimeter method because the color developed after reduction of part of the chromic acid by alcohol or ether is due to both dichromate and chromic sulfate. Furthermore, with the colorimeter method it is not possible to use the completely oxidized dichromate and the completely reduced dichromate, that is chromic sulfate, as limiting values on a calibration graph since the calibration curve of logarithm of colorimeter reading plotted against ether (or alcohol) concentration is not a straight line and the deviation is greatest at the limits.

SUMMARY

A method for determining ether (or alcohol) in blood and gas mixtures is described. The method consists of a distillation in a Widmark flask with oxidation of the transferred alcohol, or ether, by chromic acid. Excess chromic acid is titrated with ferrous sulfate, using barium diphenylamine sulfonate as an indicator.

REFERENCES

Anderson, R. K.: A Simple Method for the Determination of Alcohol in Blood, Am. J. Clin. Path. (Tech. Supp.) **6**: 85-89, 1942.

Bavis, D. F., and Arnholt, M. F.: The Specificity of Methods for Determining the Concentration of Ethyl Alcohol in Body Fluids With Specific Reference to the Heise Method, Nebraska M. J. **23**: 407-412, 1938.

Cavett, J. W.: The Determination of Alcohol in Blood and Other Body Fluids, J. Lab. & Clin. Med. **23**: 543-46, 1938.

Fisk, V. B., and Nelson, V. E.: A Micromethod for the Determination of Ethyl Alcohol in Blood, Proc. Iowa Acad. Science **48**: 207-212, 1941.

Gibson, J. G., and Blotner, Harry: The Determination of Ethyl Alcohol in Blood and Urine With the Photoelectric Colorimeter, J. Biol. Chem. **126**: 551-559, 1938.

Harger, R. N.: A Simple Micromethod for the Determination of Alcohol in Biologic Material, J. Lab. & Clin. Med. **20**: 746-51, 1935.

Kolthoff, I. M., and Sandell, E. B.: Textbook of Quantitative Inorganic Analysis, New York, 1938, The MacMillan Company, p. 472.